ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

FISCAL YEAR 1973

PART II

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

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Public Health Service National Institutes of Health

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Office of the Scientific Director

Growth and change of the NICHD intramural research program have continued in this fiscal year. The Laboratory of Cellular and Comparative Physiology, GRC, has been expanded and reorganized under a new Branch Chief, Dr. Takashi Makinodan, with the addition of a new section on Cellular Immunology added to the Branch. Dr. Makinodan and his staff are intensively pursuing new leads in the rapidly developing field of cellular immunology as related to human aging. The Section on Molecular Structure under Dr. Erhard Gross has been transferred from the Laboratory of Biomedical Sciences to the Reproduction Research Branch to facilitate closer collaboration in synthesis and structural analysis of hormones and their analogs. The Children's Diagnostic and Study Branch has been abolished. Planning has been completed and a contract awarded for construction of a new three story obstetrics and nursery unit to be added to the G wing of the Clinical Center. This will house the research and clinical activities of the Pregnancy Research Branch and a perinatology research program. The Social and Behavioral Sciences Branch has moved from space at the National Naval Medical Center to renovated facilities in the Auburn Building. The Laboratory of Biomedical Sciences and the Laboratory of Molecular Genetics have consolidated programs in newly renovated space in Building 6.

In fiscal year 1973 total budgeted positions for intramural research increased from 277 to 321. In addition a total of 106 scientists worked in NICHD laboratories as Guest Workers, Guest Scientists, and Visiting Fellows. Another 63 employees in part-time, temporary, WAE, stay-in-school, and work-study categories participated in the intramural research programs.

Developmental Immunology Branch - Accomplishments

1. Development of a means of immunization of infants against Hemophilus influenzae type b meningitis, the largest source of acquired mental retardation in the United States, remains the major research effort of scientists in this Branch. In a pilot study this year the immunogenicity of H. influenzae type b polysaccharide has been evaluated by studying the clinical condition and serum antibodies of 23 infants injected with 5-10 µg of polysaccharide when 2-3 months of age. On follow-up all injected infants had serum anti-type b antibodies well above the average level for their age, and none had levels in the nondetectable range, in contrast to 30% of uninjected infants who had no detectable anti-type b antibody at the same age. Thus, infants so treated at 2-3 months of age respond with sustained antibody levels. A controlled clinical trial of this means of immunization will begin in Mecklenburg County, N.C. in the coming year.

2. Another mechanism for inducing immunity to H. influenzae that may be simpler, more effective, and has far-reaching implications for the whole field of immunology and infectious disease, is also under study in this Branch. Strains of non-pathogenic E. coli have been isolated which have an antigen that cross-reacts with the polysaccharide antigen of H. influenzae type b. These strains of bacteria, when fed to newborn animals, produce no

illness, but do induce the formation of antibodies which protect the animal against infections due to H. influenzae. These strains are ubiquitous in nature, and probably constitute the source of the "natural" immunity to H. influenzae which most humans develop by age 6. Active immunization of all children in infancy by this "natural" means would appear to be ideal. Evidence that this method is indeed the "natural" source of immunity in humans was provided by a survey of stool cultures of all normal newborn infants discharged from the Charlotte Memorial Hospital in Charlotte, North Carolina. Of these infants, 1.1% had cultures that contained the cross-reacting E. Coli strain in the neonatal period. When reexamined at 4-13 months of age, these infants all had detectable anti-type b antibodies in the serum at levels above the average. Thus, natural acquisition of the organism in the newborn nursery, which caused neither symptoms in these infants nor interference with normal growth and development, resulted in acquisition of protective antibodies to H. influenzae type b. In infants uncolonized by these strains of E. Coli early in life antibody titres to H. influenzae at later dates were significantly lower than it was in those who were colonized.

3. Scientists in this laboratory have also identified non-pathogenic organisms with cross-reacting antigens to D. pneumoniae and N. meningitidis, raising the possibility of applying this method of immunization to diseases caused by these and other organisms as well. More extensive animal testing of this immunization technique will be carried out in the coming year, in preparation for later clinical trials.

Social and Behavioral Sciences Branch - Accomplishments

1. Study of early environmental influences on child development has demonstrated effects of father absence during infancy. The amount of father interaction with male infants was positively related to general developmental status as measured by the Bayley Mental Development Index. It was also related to the child's social responsiveness, efforts to elicit feedback from the environment, and exploratory behavior. There were no measurable significant effects of father interaction on female infants.

2. Comparison of infant caretaking patterns of mothers and surrogates showed that on the whole mothers provided higher levels of stimulation. They were more demonstrative in expressing positive affect, spent more time in non-caretaking activities with their infants, played with their infants more, and provided them with a greater variety of play objects. When assessed for general developmental status or on specific aspects of development, however, infants in surrogate care did not differ from infants cared for by their own mothers. With the growing number of working mothers, this type of study gains added importance.

Reproduction Research Branch - Accomplishments

1. Sub-units of HCG have been prepared and characterized chemically, immunologically, and biologically. Specific antisera have been prepared for each sub-unit. The sub-units were shown to be free of biologic activity. Free HCG was found in plasma throughout pregnancy, a finding which accounts

for previously noted discrepancies between biologic and immunologic measurements of HCG. The individual sub-units have been detected and levels measured in tissue, serum, and urine of patients with HCG-secreting tumors, providing evidence of cloning of cancer cells in human metastatic lesions.

2. Clinical studies of testicular regulatory mechanisms are continuing in infertile males. Serum FSH levels are high when germ cells are absent, but it has been difficult to decide whether the germ cell or Sertoli cell is responsible for regulation of FSH levels. Study of three men with a normal sperm count but only dead sperm exhibited high FSH levels, providing strong evidence that the Sertoli cell regulates FSH secretion. Additional evidence that this is the case was provided by a study in rats. When Vitamin A deficiency was used to induce reversible germ cell depletion, the FSH level did not rise, thus implicating the Sertoli cell in FSH regulation. A clinical trial has been initiated to test the effect of large doses of FSH in various types of male infertility.

3. Chromaffin granule membranes are being intensively studied as a model secretory cell membrane system. The molecular parameters of the membrane proteins have been studied by a novel system of discontinuous buffer electrophoresis in sodium dodecyl sulphur at neutral pH. X-ray diffraction studies of these membranes revealed the existence of protein particulates distributed symmetrically on the area bounded by the membrane.

4. Prolactin has been isolated from amniotic fluid, and a practical method has been devised for preparation of the hormone. The identity of serum, urine, and amniotic fluid prolactin was proved by physico-chemical, biologic, and immunologic criteria.

5. For the first time techniques have been perfected permitting isolation and numerical quantitation of lysosomes from human placentas obtained at various times during pregnancy.

Behavioral Biology Branch - Accomplishments

Section on Brain and Behavior

1. Several studies of primate vocalization have been completed. Sound spectrographic analysis of the vocalization of one pair of squirrel monkeys indicates that each animal has a distinctive fine structure in its vocal productions. Call duration, number of syllables, and complexity of syllable structure all seem to differentiate and make unique an individual monkey's vocal repertoire. Responses of single neurons in the primate auditory cortex to complex biologically significant acoustic stimuli are little affected by wide fluctuation in arousal level of the animal. The complex synaptic connections responsible for the relatively specific responses to such stimuli as species-specific vocalizations thus represent securely organized sensory channels, rather than a diffuse system related to attention and arousal.

Section on Neurobiology

1. Biochemical analytical methods developed in the Branch have been used to study the chemical secretory patterns of individual neurons of the molluscan nervous system. Synaptic inhibition was found to reduce the rate of incorporation by neurons of radioactive leucine into two low molecular weight neurosecretory proteins by 50%. Dopamine, the presumed transmitter mediating the synaptic inhibition, produced a similar reduction in synthesis of the neurosecretory protein. Synthesis rates of other proteins in the cell studied were not significantly reduced by either synaptic inhibition or dopamine. Both treatments increase membrane potential and permeability to potassium. High potassium concentrations increase permeability to potassium but decrease membrane potential, and result in a doubling of the synthesis rate of the neurosecretory protein. Thus, increase in potassium permeability is probably not the control mechanism involved; present studies are focusing on analysis of the increased membrane potential as the mechanism responsible for control of neurosecretory protein synthesis.

2. Scientists have succeeded in dissociating and establishing in tissue culture mouse cerebellar neurons, and demonstrated that these neurons develop complex patterns of spontaneous and synaptically mediated electrical activity. Clearly distinguishable morphologic types and a range of electrophysiologic characteristics were found in the surviving neuronal networks, so that some substantial representation of the relatively simple normal cerebellar circuitry appears in the culture systems. Studies are proceeding on cells from a variety of genetic mutants with well-established neuropathologic abnormalities, to attempt to ascertain at the cellular level the basis for the abnormalities.

Laboratory of Molecular Genetics - Accomplishments

1. Studies directed toward understanding the regulated expression of the globin gene have indicated that the reverse transcript of purified globin message can be used as a highly specific and sensitive probe for quantitating specific globin genes and mRNA. The number of genes corresponding to globin in several different avian and mammalian tissues, as demonstrated by this technique, is rather small, consisting of 3 to 5 globin genes per diploid genome. The number of globin genes is not amplified during the process of differentiation, so at least for globin, the gene amplification hypothesis appears excluded. Application of this technique to studying the process of differentiation in tissue culture has provided evidence that transcription of the globin genes represents a rate limiting step in differentiation, and that globin message rises from an undetectable amount to 3000-6000 molecules per cell in the fully differentiated state.

2. Continued studies of the integration and excision of phage λ into bacteria have shown that host chromosomal material can be incorporated into the phage genome in a non-random fashion. This observation provides the initial evidence that phage λ can be used as a generalized transducing probe. Examination of the genes necessary for the formation of the viral particle shows that these proteins are produced in a coordinate fashion, and suggests that there are controls at some post-transcriptional level. These results

provide a clearer picture of the process of viral insertion and excision and the elements needed for packaging the viral chromosome into immature virus particles.

Laboratory of Biomedical Sciences - Accomplishments

Section on Developmental Enzymology

1. Optimal assay conditions were ascertained and specific activities determined for a large number of placental enzymes, and zymogram techniques devised for detecting isoenzyme variation among them. Qualitative and quantitative comparisons were made of these enzyme activities in normal term placenta and malignant trophoblast in tissue culture. The tissue in culture was found to be virtually devoid of heat stable (placental) alkaline phosphatase, and had an unusual lactate dehydrogenase enzyme, differing strikingly from that of the term placenta.

Section on Intermediary Metabolism

1. Investigations in this laboratory on the biochemistry of brain transfer RNA are designed to identify aspects that are unique to brain and appear to be related to brain function. Brain mRNA has been purified and described in terms of size, poly A sequences, and ability to be translated by a protein synthesizing system. Differences have been found between the messenger fraction from the brains of immature and of adult animals with respect to the length of the poly A sequences.

Section on Physiological Controls

1. All of the enzymes known to participate in glycogen cycle phosphorylations have been partially or completely purified. Glycogen synthetase phosphatase was found to be a general phosphoprotein phosphatase that dephosphorylates phosphorylase - b kinase and other protein substrates of the cyclic AMP - dependent protein kinase. The development of fetal rat liver glycogen synthesis was reproduced in a completely defined organ culture system and shown to be related to the development of glycogen synthetase.

2. A new method for measuring tryptophan hydroxylabe was developed and used to show that this enzyme is rate-limiting in serotonin synthesis in the pineal gland and is controlled by tryptophan levels in the circulation. Norepinephrine was shown to decrease pineal serotonin levels by a sequence of reactions involving a specific receptor, cyclic AMP, and the induction of serotonin N-acetyltransferase. Antigonadotrophic effects of the pineal gland have been further delineated. A potent antigonadotrophic compound produced by the pineal gland was purified and shown to be a small peptide.

Section on Developmental Pharmacology

1. Using cells in tissue culture, which offer a considerable advantage over using the intact animal, scientists in this section have determined that induction of activity of mono-oxygenase and other drug-metabolizing enzymes by

phenobarbital, polycyclic hydrocarbons, or biogenic amines is controlled at two levels: the level of transcription involving gene activation, and a post-transcriptional level in which the normal rate of decay of induced enzyme activity is retarded. The induction process effected by phenobarbital is more dependent on ribosomal RNA synthesis than that effected by either aromatic hydrocarbons or biogenic amines.

Pregnancy Research Branch - Accomplishments

1. Scientists in this Branch have continued to evaluate the pregnant baboon as a primate model for studying the mechanisms regulating steroid synthesis and metabolism during pregnancy. Numerous studies indicate that the approximation of the human condition is quite close, and that the model will be a useful one. For example, placental utilization of estrogen precursors from the maternal circulation seems impeded in both the human and the baboon. Likewise both species demonstrate that maternal and fetal metabolism of cortisol differs from that in the non-pregnant state. Efforts are presently being made to develop and maintain a chronic preparation of the pregnant baboon, restraining the animal in a chair over a long period of time with maternal and fetal catheters in place.

2. Comparison has been made of the relative usefulness of maternal serum and urine estrogens for assessment of feto-placental status in high risk human pregnancy. The study showed that serial 24-hour urinary estrogen determinations provide the most reliable data to monitor estrogen metabolism as a criterion of fetal well-being.

3. A new accurate and sensitive radioimmunoassay for monkey glucagon has been applied to the primate model diabetic pregnancy. Glucagon levels in the mother and fetus are not affected by fasting or induced hyperglycemia in either the normal or the streptozotocin-induced glucose intolerant animal. Glucagon administered intravenously in physiologic amounts does not cross the monkey placenta in either direction. In the newborn there is a linear relation between plasma glucose and glucagon levels that is not present in the fetus.

Gerontology Research Center - Accomplishments

Laboratory of Behavioral Sciences

1. Studies of logical reasoning in elderly men in the Baltimore Longitudinal Study of Aging have shown that men over age 60 make more errors than younger men. Analysis of the errors indicates that older men are more likely to make premature attempts to synthesize information than are younger men, resulting in repeating solution attempts that have failed. These results correspond to a related study in rats, wherein older rats had much more difficulty in solving a maze than young rats, due to perseverative errors. However, when the older rats were prevented from developing the perseverative error pattern in early trials, they learned more rapidly than the young rats. Because of the similarity in type of errors, it may be possible to improve performance of the elderly humans by a training procedure similar to the one effective in the rats.

2. Work on operant conditioning of autonomic functions has continued in this laboratory. A patient with mild essential hypertension has been able to maintain her systolic blood pressure 30 mg below initial baseline levels after training. Monkeys have been able to markedly reduce their heart rates by conditioning. Several human subjects have demonstrated long-term capability of eliminating cardiac arrhythmias after conditioning. Extending the conditioning procedure to bowel control, subjects with prolonged intractable fecal incontinence have been able to achieve complete continence after operant conditioning. These studies demonstrate that conscious control over autonomic function is possible, and may have applicability in numerous areas of medicine.

Clinical Physiology Branch

1. The Baltimore Londitudinal Study of Aging continues to be a major research resource. Particular efforts this year have been directed to development of a statistical method for predicting the requisite duration of a longitudinal study, the required frequency of data acquisition, and the number of subjects needed, in order to define the rates of aging changes in selected functions with specified accuracy. These techniques greatly aid continued planning for the Baltimore study, and have application for longitudinal studies in general.

2. The incidence of cutaneous malignancies increases with age. Studies of chronic actinic damage as a precursor of these malignancies have assumed that the damaging radiation spectrum is confined to the 290-320 nm wavelengths, and that longer wavelength UV radiation (320-400 nm) protects against sun damage to skin by inducing tanning. Study in this Branch of the effect of age on sensitivity to UV light of different wavelengths has shown that, on the contrary, the longer UV wavelengths have an augmentative effect rather than a protective one. These findings indicate that present preparations to prevent sun damage should be modified so that they protect against a broader spectrum of UV light.

3. A new labeled polymeric substrate has been developed to assay renin and to aid in study of this kidney enzyme involved in the regulation of blood pressure. With this assay it has been found that renin is inhibited by a variety of proteins and peptides. The techniques and concepts developed in this laboratory should permit development of renin inhibitors of potential usefulness in clinical treatment of hypertension.

Laboratory of Cellular and Comparative Physiology

1. Observing that associated with aging and immunosenescence there is an increase in incidence of immunodeficiency diseases including autoimmunity, cancer, and infection, researchers in this Branch have been intensively studying changes in the immune system with aging. They have found, for example, that of the three cell types (T-cells, B-cells, and A-cells) involved in the initiation of a humoral immune response, the T and B cells are altered in immunologically deficient old mice, but A cells appear normal. Proliferative capacity of T and B cells decreases as much as 10-fold with age, a finding of key significance because the number of functional effector cells

generated in an immune response depends on the efficiency of their ability to divide. In addition, evidence was found that there is an age-associated increase in either the relative number or the efficiency of regulator cells which suppress the immune response, to account in another way for the reduction in immune response with aging.

2. Study of mice with an exceptionally long life span showed an age-related decline in cell-mediated immune activity and in resistance to allogeneic tumor cells. This decline had previously been observed in mice with short life spans, so that critics claimed that life-shortening diseases imposing on the immune system were responsible for the decline. The new evidence should allay this criticism.

3. A new research program in biomembrane physiology, emphasizing age effects on hormone binding, has yielded evidence that membranes from adipose tissue, skeletal muscle, brain, and prostate gland of aging rats have a progressive decline in steroid hormone binding. This decline is due in part to a reduction in the number of macromolecular binding sites per unit of tissue.

Laboratory of Molecular Aging

1. Active transport of glucose in the kidney has been studied in isolated renal brush border membranes and found to consist of at least two processes, a high-affinity binding system and a carrier-mediated transport system. Kinetic studies of the binding have characterized time course, reversibility, saturability, alterability by phlorizin, and the influence of Na^{+}, Ca^{2+}, and Mg^{2+} ions. A particulate fraction derived from detergent-extracted brush border membranes has the same affinity and capacity as intact membranes, but has lost about 90% of its protein content and shows no evidence of vesicular structures. The mechanism of uptake of high concentration of glucose by intact brush border membranes shows both influx and efflux components and is consistent with the hypothesis that there is a carrier-mediated transport into and out of vesicles. The rate of glucose transport by the membrane is greatly enhanced by a Na^{+} gradient from the outside to the inside of the membrane.

2. Relationship of the mechanism of infection to age of the cell has been studied extensively in tissue culture. Viral replication was unaffected by age of the cell, until just before cell death, when there was a decrease. Induction of interferon, the viral defense mechanism of the cell, was possible throughout the life span, but older cells required a larger stimulus to produce a given amount of interferon, indicating that the sensitivity and/or efficiency of the process declines with age. It was also found that in senescent cells the antiviral state is produced more rapidly by stimulation with the interferon inducer poly I:C than with interferon itself, suggesting either another antiviral protection mechanism in senescent cells or a change in the relative rates of uptake of poly I:C and interferon in the senescent cell. These studies may help to explain the increased susceptibility of the elderly to viral infections.

Collaborative Guest Scientist Program

1. The hypothesis that protein synthesis is impaired as age advances was examined by studying incorporation of ^{14}C-leucine or ^{14}C-labeled amino acids by liver microsomes from adult (12 month) and aged (20-31 month) female rats. Amino acid incorporation into protein was decreased by 12-43% in the cell-free system from senescent rats. Furthermore, the cytosol (105,000 g supernatant) from senescent animals inhibited protein synthesis by microsomes from the young rats, while the cytosol from the young rats combined with senescent microsomes functioned as a completely senescent system. The results support the concept that protein synthesis is impaired in senescent cells, though the inhibitory effect of cytosol from aged cells is a new finding of unknown significance.

2. Effects of age on isolated cardiac muscle function were studied in rats. Contractility of the muscle was increased by norepinephrine and paired pacing, and decreased by hypoxia. No age differences were found in response to hypoxia or paired pacing, indicating that there are no major alterations in anaerobic metabolism with aging. By contrast, at higher dose levels of norepinephrine the inotropic response was less in the old than in the young, a finding that is probably reflected in decreased "cardiac reserve", the ability to respond to increased stress, in the older animals.

Serial No. HD DB - 7
1. Office of the Scientific Director
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Specific Cellular Immunity in Recovery from Viral Infection

Previous Serial Number: Same

Principle Investigator: Lon R. White, M.D.

Other Investigator: Harrie Anne Sutton, Biologist

Cooperating Unit: None

Man Years:

Total:	0.0
Professional:	0.0
Other:	0.0

Project Description:

Objectives: To determine the role and mechanism of acquired, specific, cell-mediated immune responses in recovery from viral infection *in vitro* and *in vivo*.

Methods employed: The initial studies have utilized infection of mice with MVM (minute virus of mice) and vaccinia virus. Spleen cells from control or immunized animals have been put into culture with cells previously infected with the appropriate agent. The following variables are: a) virus replication, b) virus-induced cell injury, c) cell-induced cell injury (lymphocyte cytotoxicity).

Major findings: No evidence of such immunity has yet been demonstrated.

Significance to biomedical research and the program of the Institute: It appears likely that specific cellular immunity may play a major role in termination of viral infections *in vivo* and in the host cell injury seen in certain viral infections. It is further hypothesized that an incompetence or abnormality of this mechanism may occur when the animal is first exposed to the virus during immunologic immaturity. The *in vitro* demonstration of such phenomena would represent a major advancement in our understanding of basic immune phenomena and the pathogenesis of virus-induced diseases.

Proposed course: This project has been terminated.

Honors and Awards: None

Publications: None

Serial No. HD DB - 5
1. Office of the Scientific Director
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Immune mechanisms in chronic and congenital viral infections

Previous Serial Number: Same

Principle Investigator: Lon R. White, M.D., DB, NICHD

Other Investigators: George Nemo, Ph.D.,
Jacob A. Brody, M.D.
Harrie Anne Sutton, biologist, DB, NICHD
Samuel Baron, M.D., LVD, NIAID

Cooperating Units: Epidemiology Branch, C&FR, NINDS [NDS (CF) - 70 E 1833]

Man Years:

Total:	0.1
Professional:	0.1
Other:	0.0

Project Description:

Objectives: 1. To establish models of chronic, congenital, and/or latent viral infection in animals and in tissue culture. 2. In infected animals, to define the development of immunity to the infecting and to heterologous antigens. 3. In tissue culture, to define the effect of immunologic factors (antibodies, immune lymphocytes, interferon) on the evolution of acute and chronic infections.

Methods employed: Neonatal and pregnant mice are injected with either a reovirus type 1 or the minute virus of mice (MVM). The persistence of virus in animals is determined by isolation and fluorescent and immunofluorescent techniques. Serum levels of hemagglutination inhibition antibodies to both agents are followed. Lymphocyte transformation and cytotoxic activity is studied in spleen cell cultures in the presence of phytohemagglutinin on specific antigens. Interferon production is studied by assay of media from tissue culture preparations of cells infected with MVM, and by the ability of MVM infected cells to support the growth of other viruses. Sensitivity of MVM to interferon is tested by determining the yield of virus following exposure of cells in culture to known amounts of interferon.

Major findings: MVM does not appear to induce interferon elaboration in vitro. Pretreatment of L cells with interferon produces a mild to moderate diminution in virus yield.

Significance to biomedical research and the program of the Institute: Infection of the human embryo or fetus with rubella or cytomegalovirus is associated not only with developmental abnormalities and mental retardation, but also with infection persisting for months or years after, despite the presence of neutralizing antibody in the serum. There are several examples of related phenomena in experimental animals infected during prenatal or neonatal life. Previous studies have suggested that an impairment of the immune function of lymphocytes may be associated with such persistent infection. These phenomena provide insight into the question of how viral infection is normally terminated, and represent a challenge to older ideas on the role of specific cellular immunity in the natural history of virus infections. The investigation described represents a direct experimental approach to elucidating the cause and course of chronic infection following initial exposure to the agent during immunologic immaturity. The results of this investigation will be of immediate relevance to our understanding of other types of illness known or suspected to be associated with chronic viral infection. In addition, they may suggest new approaches to research in the role of congenital viral infection as a cause of diseases of unknown etiology such as prematurity, idiopathic growth failure, malignancy, mental retardation, developmental malformation, and autoimmune diseases.

Proposed course: To be continued.

Honors and Awards: None

Publications: Manuscript in preparation.

NOTE: Notice of Research Project (Form PHS-166) filed under the NDS Project Number. (vide supra)

Serial No. HD DB - 15
1. Office of the Scientific Director
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Search for a negative DNA strand in cells infected with the minute virus of mice (MVM)

Previous Serial Number: Same

Principle Investigator: Lon R. White, M.D., DB, NICHD

Other Investigators: Harrie Anne Sutton, Biologist, DB, NICHD
George Nemo, Ph.D.
Jacob A. Brody, M.D.

Cooperating Unit: Epidemiology Branch, C&FR, NINDS [NDS (CF) - 71 E 1922]

Man Years:

Total:	0.4
Professional:	0.4
Other:	0.0

Project Description:

Objectives: a. To demonstrate a "template" DNA (negative strand) complementary to the single-stranded DNA (positive strand) of the MVM virion. b. To determine if the negative strand is associated with the cell's chromosomal DNA. c. To investigate the kinetics of negative strand synthesis relative to cellular DNA replication, cellular division, virus infection, and virus production. d. To utilize this method to detect a latent or cryptic viral infection in the cells of experimental animals and in tissue culture. e. To characterize the virion DNA, the "negative strand," and replicative forms of MVM DNA.

Methods employed: These studies utilize radioisotope "tagged" viral DNA. Annealing with "cold" DNA from virus infected cells is demonstrated by liquid scintillation detection of double- and single-stranded DNA in fractions eluted from a hydroxyapatite column.

Major findings: Contrary to expectations, DNA extracted from purified, concentrated virus preparations is inhomogeneous and partially double-stranded. A significant amount of additional duplex formation results when the initially single-stranded DNA is allowed to "self-anneal." That these sequences are truly viral and not from the host cell is shown by the failure of added mouse or rat DNA to influence the rate or extent of annealing. Denaturation and reannealing kinetics of the initially double-stranded

suggests the presence of one or more loop or hairpin molecular configurations. Nucleic acid hybridization reactions using total cell DNA and a "stripped" (all double-stranded parts removed) viral DNA "probe," has now been used to detect complementary sequences in DNA from both acutely infected L cells (tissue culture) and newborn mice. Negative results were found with normal mouse (3 strains), rat, and guinea pig DNA, as well as with DNA from 2 tissue culture lines. Unexpectedly, positive results were found with DNA from a leukemic rat. Current work is focused on the utilization of this method to detect a latent or persistent parvovirus infection in animals and tissue culture.

Significance to biomedical research and the program of the Institute: Characterization of virus-cell interactions using a single-stranded DNA virus (parvovirus group, of which MVM is representative) is expected to advance our understanding of the mechanisms by which a virus infection might alter the genetic potential of the host cell (either a somatic or germ cell) as well as of mechanisms involved with the establishment of latent and chronic viral infections. Congenital, chronic, or latent virus infections may be involved with the etiologies of certain reproductive, developmental, and chronic diseases whose causes are now unknown. This experimental approach may prove to be directly applicable to a search for a parvovirus DNA in the cells in animals and persons with such diseases.

Proposed course: To be continued at an increased level of effort.

Honors and Awards: None

Publications: Manuscript in preparation.

NOTE: Notice of Research Project (Form PHS-166) filed under NDS PROJECT NUMBER. (vide supra)

Serial No. HD DB - 20
1. Office of the Scientific Director
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the consequences of viral infection in early life

Previous Serial Number: Same

Principal Investigator: Lon R. White, M.D., DB, NICHD

Other Investigators: Dwayne Reed, M.D.
Harrie Anne Sutton, Biologist, DB, NICHD
Jacob A. Brody, M.D.

Cooperating Unit: Epidemiology Branch, C&FR, NINDS [NDS (CF) - 72 E 1979]

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives: To investigate immune responses and neurologic development in children known or suspected of having contracted certain virus infections during infancy or early childhood. The initial studies have focused on infection with "natural" measles during the first year of life.

Methods employed: A population of approximately 100 children in and around Little Rock, Arkansas, known to have had measles before one year of age were identified with the assistance of a local hospital physician and public health officers. To test the feasibility of an intensive, longitudinal study of this cohort and control children, an attempt was made to contact, evaluate and obtain serum from several "case" and "control" children. Sera were tested for anti-measles antibody by hemagglutination inhibition.

Major findings: No "control" children could be identified. Five "cases" were examined and were considered neurologically normal although their antibody titers were slightly elevated above expected levels.

Significance to biomedical research and the program of the Institute: Arbovirus infections involving the CNS and occurring in infancy or early childhood are known to be associated with signs of minimal brain damage as the children near school age. This occurs even though the original illness was mild and followed by apparently complete recovery. Subclinical CNS involvement with the common childhood illnesses (measles, mumps, herpes

simplex, varicella, roseola infantum) is frequent; the neurologic sequelae of these illnesses are undefined but may be of considerable importance. This study represents an attempt to define the role of such early infections in the causation of minimal brain damage and related neurologic abnormalities.

Proposed course: This project has been terminated.

Honors and Awards: None

Publications: None

Serial No. HD DB - 16
1. Office of the Scientific Director
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Development and application of methods for the study of the minute virus of mice (MVM)

Previous Serial Number: Same

Principal Investigator: Lon R. White, M.D., DB, NICHD

Other Investigators: Harrie Anne Sutton, Biologist, DB, NICHD
George Nemo, Ph.D.
M.D. Hoggan, Ph.D., LVD, NIAID

Cooperating Units: Epidemiology Branch, C&FR, NINDS [NDS (CF) - 72 E 1980]
Laboratory of Viral Diseases, NIAID

Man Years:

Total:	1.8
Professional:	1.0
Other:	0.8

Project Description:

Objectives: To develop improved methods for the propagation, purification, and quantitation of the virus and its nuclei acid. To investigate basic biological, biochemical, and structural properties of the virus and its DNA in order to develop techniques to investigate its persistence in congenitally and chronically infected experimental animals.

Methods employed: The agent is propagated in tissue culture and detected by cytopathic effect or antigen production. Its DNA is radioisotope "tagged" during replication. It is purified by differential centrifugation and by chemical means. The virus, its proteins, and its DNA are characterized by velocity sedimentation in sucrose gradients, electron micrographic appearance, salt and thermal elution characteristics from hydroxyapatite columns, and polyacrylamide gel electrophoresis.

Major findings: Previous reports have described the growth of MVM in only two cell lines, both primary, with CPE in only one of these. We have extended this to 4 continuous cell lines, 2 of which show CPE. We have shown that whereas maximum virus production demands that the host cell divide, infection can occur in non-dividing cells and the virus can remain "latent" without apparent injury to the cell until it divides. In addition, we have established chronic infection in four cell lines, two of which have been

producing virus in high concentration continuously for 2 years. We have accomplished a 100-1000 x improvement in yield of virus and hemagglutinating antigen and have shown that purification by isopycnic ultracentrifugation is associated with moderate virus disruption and loss of infectivity. Additional studies have revealed no significant loss of hemagglutination or infectivity titers with 3 cycles of freeze-thawing or with heating at 56°C for up to 90 minutes. UV inactivation progresses in approximately one $\log_{10}$ decrements per second under standard conditions. The in vitro infectivity titration method has been improved from a rather unreliable one-month procedure (which works poorly with "wild" strains of MVM) to a sharply reproducible, reliable one-week procedure usable with all strains. The nuclei acid, extracted by NaOH treatment and by a pronase-phenol-chloroform method, has been found to be extremely labile and "sticky," adsorbing to plastics, millipore filters, and dialysis tubing. Results of hydroxyapatite chromatography suggest that it may have a "snap back" area where it is double-stranded, that some virions may contain some "negative" strand DNA, and that the DNA may be packaged in segments. A method has been developed for the demonstration of viral DNA in infected cells and are being used to study the course of MVM infection in vitro and in vivo. The virus has at least 3 proteins, whose molecular weights are between 50,000 and 80,000, and whose relative concentrations most closely resemble the pattern observed with a bovine parvovirus. Differences in relative concentration have been observed with cultivation in different cell lines; these are currently under study.

Significance to biomedical research and the program of the Institute: MVM is representative of a group of small, single-stranded DNA viruses (the parvoviruses) which are characterized by ubiquity, generally low virulence and cytopathogenicity, an apparent preference for dividing cells, and a high frequency of transplacental infections. The study of this agent is expected to lead to a better understanding of mechanisms and the possible role of viruses in the causation of certain genetic, reproductive, and developmental abnormalities. MVM has been relatively unstudied, presumable because of technical problems. The improved methods which we have developed, and the basic information obtained by the application of these methods now make such studies feasible.

Proposed course: To be continued at present level of effort.

Honors and Awards: None

Publications: Manuscript in preparation.

NOTE: Notice of Research Project (Form PHS-166) filed under NDS Project Number (vide supra).

NICHD Annual Report
July 1, 1972 through June 30, 1973
Developmental Immunology Branch

Summary

The immunogenicity of the Haemophilus influenzae type b polysaccharide injected into 2-3 month old infants has been evaluated by a study of the serum antibodies and clinical condition of 23 injected at 2-3 months of age. All infants have serum anti type b antibodies well above the average level for this age. None of the infants had serum antibody levels in the non-detectable range as contrasted to 30% of infants of this age who did not have anti type b antibodies detectable. Therefore, our conclusion is that 2-3 month old infants respond with sustained antibody levels following immunization with doses of 5-10 μg of the H. influenzae type b polysaccharide. A survey of stool cultures taken from normal infants discharged from the nursery at Charlotte Memorial Hospital reveal that 1.1% have an E. coli with a cross reactive antigen. In one study at ages 4-13 months, these infants all had detectable serum anti type b antibodies above the average level, indicating that natural acquisition of this organism, shown to have caused no symptoms in these infants or interference with their normal development, results in the acquisition of antibodies to H. influenzae type b.

A noncapsular antigen isolated from the H. influenzae taken from otitis media has been studied. Several protein fractions containing material reactive with H. influenzae typing serum of all six encapsulated varieties have been isolated. Immunization with this antigenic preparation induced the formation of serum antibodies that were bactericidal for H. influenzae type b and whose biologic activity could not be removed by a previous absorption with the purified capsule. The noncapsular antigen from the H. influenzae strain "Hope" absorbed bactericidal activity from several H. influenzae type b sera but could not remove this activity from antiserum prepared by injecting the cross reactive E. coli. These data indicate that a noncapsular antigen, shared by several encapsulated H. influenzae strains, can be isolated. The antigen induced the formation of serum bactericidal antibodies when the encapsulated strain was injected and, by itself, could induce the formation of serum bactericidal antibodies toward the encapsulated strain.

A protein polysaccharide conjugate of human serum albumin and the capsular polysaccharide of H. influenzae type b has been prepared. The conjugation was effected by marking an isocyanate derivative of the purified capsular polysaccharide with cyanogen bromide. The purified conjugate is currently under evaluation as an immunogen following its injection into primates.

The cellular response to H. influenzae type b capsular polysaccharide is being evaluated by the Jerne plaque technique in newborn animals fed two strains of E. coli at birth. In addition, the cellular reactivity of these fed and non-fed animals is being evaluated after a challenge dose of live H. influenzae type b. Evaluation of the cells reactive with the type b capsular polysaccharide indicates the greater sensitization in the fed animals. Further, challenge with a non-lethal dose of H. influenzae type b induces the formation of a greater proportion of cells that are reacting

with the polysaccharide in the plaque technique. These studies indicate that the cellular basis for natural immunity may be a more precise and earlier method of detection of sensitization than by simple examination of the serum anticapsular antibodies.

Two cross reacting E. coli were fed to adult primates. At a dosage level of 10^{11}, no symptoms were observed in any of the fed animals. A 4-8 fold rise in serum anti type b antibodies was observed one to two weeks after feeding. Colonization with the fed organism was transient, lasting only one month, indicating that in an adult animal, the feeding of a cross reactive antigen will induce the formation of serum antibodies to H. influenzae type b. Nine newborn primates have been fed strains Easter and 89, all animals were colonized for a variable duration period, and no symptoms or influence in growth and development were observed in the fed animals. Currently, their serum anti type b antibodies are being measured.

In collaboration with Dr. George McCracken, the University of Texas, Dallas, Texas, the Combined Study Group concerned with the antimicrobial chemotherapy of neonatal meningitis, Dr. Frits Orskov, International Escherichia Centre (WHO), Copenhagen, Denmark, and Dr. Emil Gotschlich, Rockefeller University, a survey of E. coli strains isolated from neonatal meningitis has been conducted. This survey was prompted by the observations that E. coli "K" antigens of the acidic polysaccharide capsular type was the structure most directly related with virulence rather than the more studied somatic or "O" antigen. It was found that of 44 isolates from the CSF, 38 (86%) of these E. coli have the "K1" antigen. This E. coli antigen is of great interest to the immunochemist and the biologist because of its unusual chemical and immunologic properties. This substance is serochemically identical to the capsular polysaccharide of meningococcus group B and consists of a homopolymer of sialic acid. This sialic acid, in contrast to the sialic acid polymer of the meningococcal group C polysaccharide, is neuraminidase-sensitive, resistant to HCl-catalyzed methanolysis, and is non-immunogenic when injected in the purified form. As a component of the bacteria, it is poorly immunogenic when present in the nasopharynx, injected intravenously as whole formaldehyde-treated bacteria. Thus, it would seem that this polymer, which is related to the invasive properties of Neisseria, may also confer virulence to another organism bacteria. The relation of this bacterial antigen to the immune status of the infant and mother is currently under investigation.

Currently, E. coli possessing the cross reacting antigen to the H. influenzae type b polysaccharide isolated from the stool of normal and asymptomatic infants are being collected and studied for their immunogenicity. All organisms are of the O75:H5 serotype and none has been associated with disease symptoms in human infants.

A symposium was conducted in collaboration with the National Institute of Allergy and Infectious Diseases to review mechanisms for the induction of immunity to polysaccharides by rendering these polysaccharides immunogenic. One of the more pressing objectives of the conference was to reach an understanding of the immunogenicity of polysaccharides and a characterization of the biosynthesis of antibodies to these polysaccharides in infants and

children. Publication of the symposium should provide a collection of information related to the field of preventive immunization for invasive diseases, especially meningitis, for the first time and should serve as a source of information and inspiration for young people entering the field of science.

Serial No. HD-I-4 (c)
1. Developmental Immunology Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Identification of non-pathogenic bacterial antigens cross reactive with meningococcal and pneumococcal capsular structures.

Principle Investigator: John B. Robbins, M.D.

Other Investigators: Rachel Schneerson, M.D.
Emil C. Gotschlich, M.D.
Teh-Yung Liu, Ph.D.

Cooperating Units: Rockefeller University, New York, N.Y.
Brookhaven National Laboratory, Long Island, N.Y.

Man Years: Total: 24/12
Professional: 24/12
Other: None

Project Description:

Objectives: "Natural" and protective serum antibodies to the capsular polysaccharide of N. meningitidis Group A have been detected in about 70% of children and young adults despite the scarcity of nasopharyngeal isolates or disease caused by this organism in the United States for the past 20 years. Further, the wide prevalence of anti capsular antibodies to N. meningitidis Group C, D. pneumoniae types I and III, and H. influenzae type b is not readily explained by asymptomatic carriage of the homologous organism. Accordingly, a search was conducted for non-pathogenic bacteria with similar polysaccharide structures as the capsular polysaccharides for these pyogenic organisms to provide an explanation for the "natural" antibodies to these antigens as well as to consider such cross reacting bacteria as possible immunogens or vaccines.

Methods Employed: Approximately 5,000 bacteria from serotypically defined collections from the CDC, Atlanta, Ga., WHO Escherichia Reference Centre, Copenhagen, Denmark, and the collection of defined bacilli of Dr. Ruth Gordon, Rutgers University, New Brunswick, N.J., have been analyzed for cross reacting antigens by immunodiffusion with appropriate antisera. Cross immunogenicity has been analyzed by intravenous injection of the bacteria and analysis of the resultant serum by quantitative and biological assays such as protection tests.

Major Findings: Several naturally occuring Bacillus pumilis as well as 7 of the 20 strains of the collection of Dr. Ruth Gordon were found to possess a cross reacting antigen to the meningococcal Group A polysaccharide. The cross reacting from all these B. pumilis strains was immunologically identical and the chemical structure was shown to consist of N-acetyl-mannosamine phosphate, mucopeptide and glycerol phosphate. The immunodominate antigen

was found to be N-acetyl-D-Mannosamine phosphate.

Four E. coli with a cross-reacting antigen to the Group C meningococcal polysaccharide have been found. The cross-reacting antigen is a homopolymer of sialic acid that is susceptible to neuraminidase and is cleaved by HCl-catalyzed methanolysis indicating its resemblance to both the Group B and Group C meningococcal polysaccharides. Immunization of animals with both the Group A and Group C cross reactants raised precipitating antisera to the appropriate antigen and this precipitating antiserum has specific group protective activity.

11 E. coli and one strain of Bacillus cereus (ATCC No. 10201) were detected with cross reacting antigen to the pneumococcal type III polysaccharide. Both cross reacting antigens contained glucuronic acid and both the E. coli and B. cereus elicited the formation of anti type III antibodies when injected into rabbits.

Significance of this research - Structural analogs that are immunogenic have been detected to the capsular polysaccharides of pyogenic bacteria. In many cases, these enteric organisms are responsible for the age-related development of "natural" and protective antibodies to these bacteria.

Honors and Awards: None

Publications:

Robbins, J.B., Myerowitz, R.L., Whisnant, J.K., Argaman, M., Schneerson, R., Handzel, Z.T., and Gotschlich, E.C.: Enteric Bacteria Cross-Reactive with Neisseria meningitidis Groups A and C and Diplococcus pneumoniae Types I and III. Infect. Immun. 6:651-656, 1972.

Robbins, J.B., Gotschlich, E.C., Liu, T.Y., Schneerson, R., Handzel, Z.T., Argaman, M., Parke, Jr., J.C., and Myerowitz, R.L.: Bacterial Antigens Cross Reactive with the Capsular Polysaccharides of Haemophilus influenzae type b, Neisseria meningitidis Groups A and C and Diplococcus pneumoniae types I and III. Proc. Symposium on New Approaches for Inducing Natural Immunity to Pyogenic Organisms, March 1973 (in press).

Serial No. HD-I-8
1. Developmental Immunology Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Identification of the immune response to Haemophilus influenzae type b capsular polysaccharide induced by neonatal feeding of bacteria with cross reacting antigens.

Previous Serial Number: Same

Principle Investigators: Zeev Handzel, M.D.
Richard Myerowitz, M.D.
Rachel Schneerson, M.D.
John B. Robbins, M.D.

Other Investigators: None

Man Years: Total: 12/12
Professional: 12/12
Others: None

Project Description:

Objectives: To identify the effect upon "natural" immunity to Haemophilus influenzae type b by feeding neonatal rabbits and adult and neonatal primates with bacteria containing cross reacting antigens to the capsular polysaccharide of Haemophilus influenzae type b, and to follow human newborns with "natural" infections of cross reacting bacteria with respect to their growth and development and H. influenzae type b antibody formation.

Methods Employed: Neonatal animals have been fed two E. coli strains designated 075:H5 which contain a newly described cross reacting antigen to the type b polysaccharide. Colonization with these organisms was determined by culturing the stool and nasopharyngeal area with antiserum agar plates. Measurement of serum antibodies to the type b capsular polysaccharide as well as to E. coli antigens was done by radioimmunoassay and a passive hemagglutenation test.

Significance of Work: Induction of "natural" immunity to Haemophilus influenzae type b by neonatal feeding of a cross reacting, non-pathogenic bacteria, might provide an alternate method for immunization against diseases caused by this organism. In addition, since analogs to the capsular polysaccharides toward other pyogenic bacteria including meningococci and pneumococci have been found, this method, if proved to be safe and immunogenic, might provide a general method for the artificial and accelerated induction of "natural" immunity toward bacteria that cause meningitis. Such an immunization program would offer a simple method, independent of the inhibition effect of maternal serum antibody, of early and widespread immunization against bacterial meningitis.

Honors and Awards: None

Publications:

Robbins, J.B., Schneerson, R., Argaman, M., and Handzel, Z.T.: Haemophilus influenzae type b: Disease and immunity in humans. Ann. Intern. Med. 78:259-269, 1973.

Myerowitz, R.L., Handzel, Z.T., Schneerson, R., and Robbins, J.B.: Induction of Haemophilus influenzae type b capsular antibody in neonatal rabbits by gastrointestinal colonization with cross-reacting Escherichia coli. Infect. Immun. 7:137-140, 1973.

Robbins, J.B., Handzel, Z.T., Schneerson, R., Parke, J.C., Jr., Argaman, M. and Robbins, J.B.: Heteroimmunization of primates to H. influenzae type b capsular polysaccharide (HITB) by gastrointestinal colonization with a non-enteropathogenic E. coli (075:K147:H5 strains Easter and "89") possessing a cross reacting capsular antigen (CRA) to HITB. Society for Pediatric Research (in press).

Serial No. HD-I-9
1. Developmental Immunology Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of Escherichia coli Isolated from Cases of Neonatal Meningitis.

Previous Serial Number: None

Principle Investigators: John B. Robbins, M.D.
George H. McCracken, M.D.
Emil Gotschlich, M.D.
Frits Orskov, M.D.
Lars Hanson, M.D.

Cooperating Units: The University of Texas Southwestern Medical School At Dallas;
The Rockefeller University, New York City;
The Escherichia Reference Centre (WHO), Statenserum-institut, Copenhagen, Denmark;
University of Goteborg, Department of Immunology, Goteborg, Sweden.

Man Years: Total: 5/12
Professional: 5/12
Others: None

Project Description:

Objectives: To study the "K" or capsular polysaccharide antigens of E. coli isolated from cases of neonatal meningitis with antisera to known pyogenic organisms. To observe if the serologic properties of capsular polysaccharides of known meningitis organisms such as Haemophilus influenzae type b, Neisseria meningitidis Groups A,B, and C and Diplococcus pneumoniae types I and III.

Methods Employed: Dr. George McCracken is the Director of a Combined Study Group concerned with evaluation of the antimicrobial chemotherapy of neonatal meningitis. E. coli isolated from these cases is studied by immunodiffusion with antisera to the above mentioned pyogens. Mouse virulence assays and counterimmunoelectrophoresis for capsular antigen in blood and CSF. Serotyping for H and O antigens are done by described methods using reagents at the E. coli Reference Centre.

Major Findings: Of 67 E. coli isolates from neonatal meningitis, 58 isolates have come directly from the CSF. 50 of these E. coli have a capsular polysaccharide that is serochemically identical to the Group B meningococcal polysaccharide. This E. coli structure is a homopolymer of sialic acid that is susceptible to hydrolysis with neuraminidase. The purified polysaccharide in contrast to the other capsular polysaccharides of meningococci,

pneumococci, and H. influenzae type b is non-immunogenic. The E. coli capsular antigen was found to be independent of somatic or flagella antigens.

Significance of this Research: A virulence factor has been found for neonatal meningitis due to E. coli. Such questions as the role of placentally transmitted antibody, colostral antibody, maternal or surrounding flora, may be investigated to understand the pathogenesis of this disease and, perhaps, to design preventive measures.

Honors and Awards: None

Publications: None

NICHD Annual Report
July 1, 1972 through June 30, 1973
Developmental Immunology Branch
Contract and Collaborative Research

Contract Title: Development and Testing of Capsular Polysaccharide of Haemophilus influenzae type b

Contractor: Charlotte Memorial Hospital
P.O. Box 2554
Charlotte, North Carolina

Money Allocated: $38,000

Objectives: To confirm the immunogenicity of the type b polysaccharide in infants and children. To study the optimum dose (serum antibody response) in infants and children. To determine the relationship between maternal serum antibody and the immune response to the type b capsular polysaccharide. To determine the effect upon the immune response to the type b capsular polysaccharide of the presence of bacterial antigens with cross reactive moieties to the type b capsular polysaccharide. To determine the overall incidence, the age distribution, and other factors such as economic and racial makeup of infants who have contracted Haemophilus influenzae type b diseases in Mecklenburg County.

Methods: To determine the optimum immunogenic dose of type b polysaccharide for infants as well as to study the effect of the immune response of the type b polysaccharide conferred by bacterial antigens with cross reacting antigens to the Haemophilus influenzae type b polysaccharide, all infants who leave the newborn nursery at Charlotte Memorial Hospital will have their stool cultures examined for cross reacting antigens by the anti serum agar technique and their level of anti type b antibodies as studied in the cord serum and maternal serum will be measured by the radioassay. When these infants return to Charlotte Memorial Hospital to participate in their continuing care program, the parents of two to three month old infants appearing for their first DPT injection will be interviewed and requested to have their infants particpate in the immunization program. Blood samples, nasopharyngeal cultures, and rectal swabs will be obtained at one, two, three and six months following injection of the polysaccharide. Untoward reactions following the injection are to be studied by phoning the parents of the child within 24 hours following injection.

A survey of the prevalence of cross reacting antigens and the effect of non-typable Haemophilus influenzae upon the immune response to the type b polysaccharide is also being studied. Four-day old infants with cross reacting bacterial antigens isolated from their stool will be studied for the sequential appearance of anti type b antibodies "natural" immunity, as compared to controlled infants and children without demonstrable cross reacting bacteria.

Honors and Awards: None

Publications:

Parke, J.C., Jr., Schneerson, R., Robbins, J.B.: The attack rate, age incidence, racial distribution, and case fatality rate of Haemophilus influenzae type b meningitis in Mecklenburg County, North Carolina: J. Pediat. 81: 765-769, 1972.

Robbins, J.B., Parke, J.C., Jr., Schneerson, R., and Whisnant, J.K.: Quantitative Measurement of "Natural" and Immunization-induced Haemophilus influenzae type b Capsular Polysaccharide Antibodies. Pediat. Res. 7:103-110, 1973.

NICHD Annual Report
July 1, 1972 through June 30, 1973
Social and Behavioral Sciences Branch

Summary of Research Activities

In progress are several related studies concerned with fundamental issues in early development: the measurement of temperament in early infancy; the analysis of the early environment and its influence on development; the role of the father in infant care and his influence on the child's development; the development of focused relationships during the first year; the infant's selective impact on his environment, how his characteristics determine the effective environment by selectively eliciting and responding to stimulation. Many of these studies are using the same sample at different developmental periods.

In the study of temperamental characteristics, we are assessing individual differences at three days in irritability, consolability, alertness, responsiveness to visual and to auditory stimuli. We are interested in determining whether there is any consistency in these characteristics between three days and four weeks and whether there is any predictability to characteristics assessed at six months. The main instrument being used is the Brazelton Neonatal Assessment Scale. In a study of the clinical usefulness of the Scale we found that it distinguishes infants of mothers on methadone from normal infants. Methadone infants at three days were found to be more irritable, less alert and less responsive to visual stimuli. These findings were presented on a symposium on the Brazelton Neonatal Assessment Scale at the meetings of the Society for Research in Child Development, March 1973.

A major aspect of this investigation of infant temperament is concerned with analyzing the infant and his early environment as a dynamic interactional system. We are not simply concerned with the effects of the environment on the infant, but are interested in how the infant influences the patterns of stimulation he receives from his caretakers. For example, we are testing the hypothesis that the amount of stimulation an infant receives may be dependent on his level of alertness and responsiveness. We are looking closely at patterns of maternal behavior with the infant at four weeks in an effort to determine the extent to which maternal behavior towards the infant is influenced by his characteristics. We are investigating whether discrepancies between mother and infant in such characteristics as activity level are associated with disturbances in mother-infant adaptation. For this study we have extended our mother-infant interaction observation schedule downward and have developed an observational technique for studying mothers and four week infants. This schedule includes a more elaborate approach for studying interaction contingencies, and has more differentiated categories for measuring tactile and kinesthetic stimulation. Another important addition to the scale is a set of categories to take into consideration the infant's state during his interactions with his mother.

A third study with this sample is focusing on the role of the father with the young infant, his direct interactions with the infant, his emotional support to the mother, and his influence as mediated by the mother. Observations of father-infant interaction are being made in the home, and interviews are being conducted with the father. This study's significance lies in its concern with an area largely neglected by other investigators.

The infants assessed neonatally are being studied again at six months. There are several aspects to this study. One is to determine whether there is any predictability from neonatal temperamental characteristics to cognitive and cognitive-motivational characteristics assessed at six months. Another issue we are looking at is the impact of the very early mother-infant relationship and patterns of stimulation on development at six months.

With the same middle-class sample, we are replicating an investigation of early environmental influences on development that was carried out with infants from lower socioeconomic homes. All too frequently important implications for theory and for practice are drawn from data based on a few studies in restricted experimental or natural situations or in which the sample is limited to one cultural group. In order to be able to make meaningful generalizations about the significance of specific aspects of early experience for development, it seems important to examine the relationships in a variety of environmental contexts.

Also in progress is an investigation of "attachment" at one year of age. Although the study of attachment is central to understanding the development of the capacity for meaningful interpersonal relationships, the major indices that have been used in studies of attachment are fear of strangers and protest on being separated from the mother. Most of the research on attachment has been carried out in contrived situations of a rather stressful nature. There is as yet little understanding of the factors that influence the quality of the reciprocal affectional relationship. In this study we are trying to assess the factors in the mother and in the infant which affect this relationship. We are also comparing indices of attachment in experimental situations with observations of infants' reactions to similar situations occurring naturally in the home. Underlying this methodological question is the conceptual issue of what are the most appropriate behavioral measures of this relationship. A theoretical paper on some issues in the conceptualization of attachment and the choice of criteria for measuring attachment was published this year.

The impact of compensatory educational programs such as Headstart or the effects of day care programs on the development of young children are problems of great practical import. The measures that have been used to evaluate these programs have been largely restricted to tests of intellectual development; very little has been done to evaluate the impact of these programs on the child's social and motivational development. This year we have been part of a collaborative study with the University of Florida, Temple University and Syracuse University to develop and test out motivational and social measures, such as persistence and trust, in young children from six months to three years of age.

In preparation is a book on the methodology and substantive findings of the investigation of early environmental influences on personality and cognitive development of infants from homes of lower socioeconomic status. This comprehensive report presents the differential effects on infant development of components of maternal stimulation and of specific aspects of the inanimate environment. Several theoretically meaningful relationships have been found between aspects of stimulation and infant development. For instance, kinesthetic stimulation had significant relationships with many aspects of development, while responsiveness of inanimate objects had a more selective effect; it was most highly related to secondary circular reactions, the infant's efforts to elicit feedback from the environment. The relevance of these findings for several important theoretical formulations are being discussed in detail. In addition to the significance of these findings for theories of early development, we expect that the techniques developed for home observations will be useful contributions to methodology. Several investigators have already adapted these techniques for a variety of research problems.

Analyses of data from this study on other aspects of early environmental influences have yielded some interesting findings with regard to the effects of father absence during infancy and substitute caretaking by surrogates in families where the mother is working. With regard to father influences it was found that the amount of father interaction with boy infants was significantly related to general developmental status as measured by the Bayley Mental Developmental Index; it was also significantly related to social responsiveness, to an index of secondary circular reactions and to measures of the infant's exploratory behavior with novel objects. There were no significant effects on the functioning of girl infants. These data were presented in a paper at the meetings of the Society for Research in Child Development.

With the ever growing number of working mothers, questions regarding the effects of surrogate caretaking are becoming increasingly important. In a paper presented at the meetings of the Society for Research in Child Development in Philadelphia, data comparing the caretaking patterns of mothers and surrogates were given. On the whole, mothers provided higher levels of stimulation than the surrogates. They were more demonstrative in expressing positive affect, they spent more time in non-caretaking activities with their infants, they played with their infants more and provided them with a greater variety of play objects. Infants in surrogate care, however, did not differ from infants cared for by their own mothers in general developmental status or on specific aspects of development.

Members of the staff have continued to serve on committees of professional societies, on the editorial boards of journals and have provided consultation to a variety of organizations.

Dr. Frank Pedersen has been on a committee of the Society for Research in Child Development which has developed a code of ethical principles for

research with young children and their parents. This statement of principles has been accepted by the Governing Council of the Society and will be published in the Directory of the Society.

Dr. Pedersen has given consultation to Station WGBH-TV in Boston regarding the content of a program which will focus on the role of the father in psychological development. This program will be moderated by Dr. T. Berry Brazelton.

Dr. Robert Klein and Dr. Frank Pedersen have continued to provide consultation to the Bureau of Standards which is assisting in setting industry standards for toy safety.

Dr. Frank Pedersen participated in a workshop on "Research in Early Childhood" sponsored by the District of Columbia Psychological Association at Williamsburg, Virginia, November 11-12, 1972. He discussed environmental factors affecting early development and presented some of the data from our study, "Environmental Influences on Cognitive and Personality Development in Infancy."

Dr. Robert Klein is serving as a consultant on statistical design to the Center for Policy Review of Catholic University.

Dr. Leon Yarrow has just completed a two year term as President of the Society for Research in Child Development, an interdisciplinary society composed of behavioral, social and biological scientists concerned with human development. Dr. Yarrow organized a Presidential Symposium for the biennial meetings of the Society in March 1973 that dealt with issues of basic and applied research in child development and the mutually beneficial interactions between theory and application.

Dr. Yarrow continues to serve on the Governing Council of the Society for Research in Child Development. He is currently a member of a joint committee of the American Academy of Pediatrics, the Academy of Child Psychiatry and the Society for Research in Child Development on planning interdisciplinary symposia and conferences for the meetings of these societies. He is a member of the editorial boards of the Merrill-Palmer Quarterly Journal of Human Development and an editorial consultant to the Monographs of the Society for Research in Child Development.

Dr. Yarrow presented lectures to the Psychology Department of the University of Virginia in Charlottesville and to the Child Psychiatry Division of Children's Hospital Washington, D. C. on the differentiation of the early environment and its selective impact on infant development. Dr. Yarrow was an invited discussant in a conference on "The Origins of Behavior" sponsored by the Educational Testing Service in Princeton, New Jersey. At the midwinter meetings of the American Psychoanalytic Association in New York he was a discussant on a workshop, "Psychoanalytic Perspectives on Programs for Young Disadvantaged Children." He is also an invited participant in

a conference on "Cultural and Social Influences in Infancy and Early Childhood" sponsored by the Wenner-Gren Foundation for Anthropological Research at Burg Wartenstein, Austria, June 18-26, 1973.

There have been several changes in staff during the past year. Dr. George Morgan joined the staff of the Social and Behavioral Sciences Branch as a senior staff fellow in February. Dr. Michael Duchowny became a staff member in July 1972 as a Research Associate. Dr. William Davidson completed his assignment as Research Associate in June 1972. Ms. Florine Carpenter resigned as secretary. Ms. Alice Frances came on duty as secretary, July 1972.

Papers Presented at Professional Meetings:

Copans, S. A. "Clinical Impressions of Communal Childrearing." Annual Meeting of the American Psychological Association, Honolulu, August 1972.

Morgan, G. A. "Determinants of Infants' Reactions to Strangers: Effects of Age and Situation." Biennial Meeting of the Society for Research in Child Development, Philadelphia, March 1973.

Pedersen, F., Rubenstein, J. and Yarrow, L. "Father Absence in Infancy." Biennial Meeting of the Society for Research in Child Development, Philadelphia, March 1973.

Rubenstein, J., Pedersen, F. and Yarrow, L. "A Comparison of Maternal and Surrogate Caretaking Behavior of Five-Month-Old Infants." Biennial Meeting of the Society for Research in Child Development, Philadelphia, March 1973.

Soule, A. B., Standley, K., Copans, S. and Davis, M. "Clinical Implications of the Brazelton Scale." Biennial Meetings of the Society for Research in Child Development, Philadelphia, March 1973.

Standley, K. and Soule, A. B. "Women in Professions: Historic Antecedents and Current Lifestyles." Annual Meeting of the American Psychological Association, Honolulu, September 1972.

Yarrow, L. "Cognitive and Motivational Development in Early Childhood." Annual Meeting of the American Psychological Association, Honolulu, September 1972.

Yarrow, L. Pedersen, F. and Rubenstein, J. "Mother-Infant Interaction and Development in Infancy." An invited paper to be presented at the Wenner-Gren Conference on Cultural and Social Influences in Infancy and Early Childhood at Burg-Wartenstein in Austria, June 1973.

Publications:

Brown, W.: A prospective study of post partum psychiatric disorders. In Morris, N. (ed.): Psychosomatic Medicine in Obstetrics and Gynaecology. Basel, Switzerland, S. Karger, 1972, pp. 350-354.

Brown, W., Manning, T. and Grodin, J.: Prenatal psychological state and the use of drugs in labor. American Journal of Obstetrics and Gynecology. 113: 598-601, 1972.

Brown, W., Manning, T. and Grodin, J.: The relationship of antenatal and perinatal psychological variables to the use of drugs in labor. Psychosomatic Medicine. 34: 119-127, 1972.

Copans, S. A.: Human prenatal effects: Methodological problems and some suggested solutions. Merrill-Palmer Quarterly. In press.

Morgan, G. A. and Ricciuti, H. N.: The Morgan-Ricciuti method. In Simon, A., Boyer, E. G. and Karafin, G. R. (eds.): Anthology of Early Childhood Observation Techniques. Philadelphia, Research for Better Schools. In Press.

Morgan, G. A.: Fear of strangers in human infants. In Brockman, L. M., Whiteley, J. H. and Zubek, J. P. (eds.): Child Development: Selected Readings. Toronto, McClelland and Stewart Ltd. In press.

Soule, A. B. and Standley, K.: Lawyers' perceptions of sex discrimination in their profession. Journal of American Bar Association. In press.

Standley, K. and Soule, A. B.: Women and architecture. Journal of Architectural Education. In press.

Standley, K. and Soule A. B.: Women in male-dominated professions: Contrasts in their personal and vocational histories. Journal of Vocational Behavior. In press.

Yarrow, L. J.: Attachment and dependency: A developmental perspective. In Gewritz, J. L. (ed.): Attachment and Dependency. Washington, D. C., V. H. Winston & Sons, Inc., 1972, pp. 81-95.

Yarrow, L. J.: Enrichment and deprivation: Towards a conceptual and empirical differentiation of the early environment. In Monks, F. J., DeWitt, J. and Hartup, W. (eds.): Determinants of Behavioral Development. New York, Academic Press, Inc., 1972, pp. 313-329.

Yarrow, L. J. and Klein, R. P.: Summary of research with the Inventory of Children's Preschool Experiences. In Simon, A., Boyer, E. G. and Karafin, G. R. (eds.): Anthology of Early Childhood Observation Techniques. Philadelphia, Research for Better Schools. In press.

Yarrow, L., Sklar, S., Pedersen, F., Lomonaco, S. and Fox, D.: Inventory of Children's Preschool Experiences. In Simon, A., Boyer, E. G. and Karafin, G. R. (eds.): Anthology of Early Childhood Observation Techniques. Philadelphia, Research for Better Schools. In press.

Serial No. HD-GD3(c)
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of Preschool Experiences on the Intellectual and Personality Development of Children from Culturally Deprived Backgrounds

Previous Serial Number: Same

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Robert P. Klein, Ph. D., Frank A. Pedersen, Ph. D., Kay Standley, Ph. D., Sandra Sklar, M.A., Bette Marcus, M.A.

Cooperating Units: Office of Economic Opportunity, Project Headstart, National Capital Day Care Association

Man Years:

Total:	.75
Professional:	.45
Other:	.30

Project Description:

Objectives: This study has been concerned with the impact of early preschool experiences on the development of cognitive skills, behavioral controls and self-concept in four-to-five-year-old children from culturally disadvantaged backgrounds. A major goal has been to develop methods for differentiated analyses of preschool learning environments, utilizing concepts from social learning theory, cognitive developmental theories and operant learning theory.

Methods Employed: The sample consisted of 58 children in 12 preschool classrooms in Headstart and day care programs. Time-sampling observational techniques were used to analyze the preschool environment. To provide a sample of different classroom activities and minimize effects of day-to-day changes, each child was observed for 20 observational units on each of three days in a balanced order. These observations were made on each child at three different points in the school year--in the fall, shortly after the beginning of school; in the middle of the term; and in the early summer,

toward the end of the term. A comprehensive observational system was developed. This system is organized in terms of the following major categories: teacher behavior, which include dimensions of teacher control, cognitive input dimensions, the affective qualities of the interaction, and evaluative feedback; child-behavior variables, focusing on self-initiated or self-directed activities; and background and setting variables.

Measures of the dependent variables consisted of several standard tests and others developed especially for this investigation. For studying cognitive functions, the following techniques were used: the Wechsler Preschool Scale of Intelligence, several measures of language functions, the Johns Hopkins Perceptual Test, selected items from the Frostig Developmental Test of Visual Perception, and a specially designed object categorization test for assessing concept development. As measures of "behavioral controls," we have adapted the Maccoby motor inhibition measures, and have developed techniques to assess capacity to delay gratification, a measure of focused attention, and a situational test of response to a difficult task. In addition, we have developed a self-concept test designed to tap various aspects of the child's feelings about himself, his competencies and deficiencies, and his perception of how he is evaluated by other people.

Major Findings: Analyses of the data have been completed. To assess the relationships between the child's experiences in the classroom and changes in general intellectual level, in specific cognitive functions, and on his self-concept, simple correlational analyses and multiple regression analyses were carried out.

The major findings have been reported in previous annual reviews. Essentially there were few significant relationships between specific experiences in these preschool programs and cognitive and personal-social characteristics.

Significance to Biomedical Research and the Program of the Institute: Current controversy about the relative merits of various approaches to preschool programs emphasizes the need for greater conceptual clarity about the preschool environment. The concepts and techniques developed in this research should contribute to more adequate methodologies for studying complex environmental inputs in relation to the cognitive and personal-social development of preschool children.

Proposed Course of Project: All analyses have been completed. It is hoped that current data collection activities on other projects will allow time to complete the final report by the middle of next fiscal year.

Honors and Awards: None

Publications:

Yarrow, L. J. and Klein, R. P.: Summary of research with the Inventory of Children's Preschool Experiences. In Simon, A., Boyer, E. G. and Karafin, G. R. (eds.): Anthology of Early Childhood Observation Techniques. Philadelphia, Research for Better Schools. In press.

Yarrow, L., Sklar, S., Pedersen, F., Lomonaco, S. and Fox, D.: Inventory of Children's Preschool Experiences. In Simon, A., Boyer, E. G. and Karafin, G. R. (eds.): Anthology of Early Childhood Observation Techniques. Philadelphia, Research for Better Schools. In press.

Serial No. HD-GD4 (c)
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Environmental Influences on Cognitive and Personality Development in Infancy

Previous Serial Number: Same

Principal Investigators: Leon J. Yarrow, Ph. D., Judith L. Rubenstein, Ph.D., Frank A. Pedersen, Ph. D.

Other Investigators: Myrna Fivel, M.A., Joan Durfee, M.A., Richard Cain, M.Ed.

Cooperating Units: D. C. Department of Public Health, Children's Hospital of the District of Columbia, Group Health Association of Washington, D. C.

Man Years:

Total:	1.10
Professional:	1.00
Other:	.10

Project Description:

Objectives: In this investigation of early environmental influences on cognitive and personality development during the first year of life, one major goal has been to develop an observational instrument to analyze the components of the early environment. A second objective was to study relationships between specific parameters of the environment and specific aspects of development during the first six months.

Methods Employed: The original sample consisted of 70 infants and their caretakers obtained through two well-baby clinics in the District of Columbia and through a private medical care program. Pediatric and neurological examinations were conducted to exclude infants with abnormalities that might be associated with developmental retardation. For the major analyses we selected from the original sample 41 cases in which we felt there was a representative sample of the infant's early experiences. These were cases in which there was a caretaker who had been responsible for the baby for three months or longer, and in which there had been no abrupt environmental changes around the observation period.

Data on environmental variables were obtained through time-sampling observations in the home when the infants were five months of age. Measures of infant characteristics were obtained by means of the Bayley Scales of Infant Development and a situational measure of exploratory behavior and preference for novel stimuli.

Major Findings: The major findings have been summarized in previous annual reports. A number of significant relationships were found between specific parameters of the environment and differentiated aspects of infant functioning. During the past year we have concentrated on the preparation of a detailed report on the findings and their theoretical and methodological implications for publication as a book. In addition two papers on discrete aspects of the study have been presented at the meetings of the Society for Research in Child Development. A paper, "Mother-Infant Interaction and Development in Infancy," has been prepared for presentation at a conference on Social and Cultural Influences in Infancy and Early Childhood sponsored by the Wenner-Gren Foundation to be held June 18-26, 1973 at Burg Wartenstein, Austria.

The first paper, "A Comparison of Maternal and Surrogate Caretaking Behavior of Five-Month-Old Infants," presented at the Society for Research in Child Development meetings by Judith Rubenstein has important implications for the ever increasing number of working mothers whose children have substitute caretakers for part or all of the day. The mothers and surrogates were compared on eleven measures of the environment which previous analyses had indicated were significantly related to important aspects of infant development. Mothers provided more stimulation than the surrogates on all eleven measures; five of these differences were statistically significant. Mothers were more demonstrative than surrogate caretakers in the expression of positive affect, in the amount of time they spent playing with their infants and in providing them with play objects; they gave their infants more varied social experiences and a greater variety of inanimate objects. These differences in patterns of stimulation did not, however, seem to affect infant functioning. Infants in surrogate care, on the whole, did not differ from infants cared for by their own mothers in general developmental status or on specific sectors of development.

The second paper, "Father Absence in Infancy," presented by Frank Pedersen at the meetings of the Society for Research in Child Development compared infants reared in father absent homes with infants reared in homes in which the father was present. Forty-nine per cent of the sample recruited from public well-baby clinics were from father absent homes. Infants from father absent and father present homes were compared on sixteen measures of cognitive, social, motor and motivational development. We also obtained from the mother an estimate of the amount of the father's interaction with the infant.

A number of interesting findings emerged. Although father absence has no relationship to functioning in female infants, several significant relationships were found in the male infants. Amount of father interaction

with boy infants is significantly related to the Bayley Mental Developmental Index, to the Bayley clusters, Social Responsiveness, and Secondary Circular Reactions, and two measures of exploration of novel objects. We checked carefully the possibility that these findings might be artifactual, that they might be systematically related to other factors, i.e., differences in maternal behavior or socioeconomic status--and eliminated these possibilities. These data do not support the prevalent assumption that maternal variables alone are the only important influence on infant development.

Significance to Biomedical Research and the Program of the Institute: This research has significance for theories of early development and has implications for early child care programs. In addition, the techniques developed for home observations will be useful contributions to methodology of studying the early environment.

Proposed Course of Project: It is planned to report the major findings in book form and terminate this study early in the next fiscal year.

Honors and Awards: None

Papers Presented at Professional Meetings:

Pedersen, F., Rubenstein, J. and Yarrow, L.: Father absence in infancy. Presented at the Biennial Meeting of the Society for Research in Child Development, Philadelphia, Pa., March 1973.

Rubenstein, J., Pedersen, F. and Yarrow, L.: A comparison of maternal and surrogate caretaking behavior of five-month-old infants. Presented at the Biennial Meeting of the Society for Research in Child Development, Philadelphia, Pa., March 1973.

Yarrow, L.: Cognitive and motivational development in early childhood. Presented at the annual meeting of the American Psychological Association, Honolulu, September 1972.

Yarrow, L., Pedersen, F. and Rubenstein, J.: Mother-infant interaction and development in infancy. An invited paper to be presented at the Wenner-Gren Conference on Cultural and Social Influences in Infancy and Early Childhood in Austria, June 1973.

Publications:

Yarrow, L. J.: Enrichment and deprivation: Towards a conceptual and empirical differentiation of the early environment. In Monks, F. J., DeWitt, J. and Hartup, W. (eds.): Determinants of Behavioral Development. New York, Academic Press, Inc., 1972, pp. 313-239.

Serial No. HD-SB1(c)

1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: An Observational Approach to the Measurement of Preschool Environments

Previous Serial Number: Same

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Robert P. Klein, Ph. D., Sandra Sklar, M.A., Alice Abramson, M.A.

Cooperating Units: Montessori Society of Chevy Chase, Bank Street College of Education

Man Years:

Total:	.50
Professional:	.50
Other:	

Project Description:

Objectives: This research is concerned with refinement and further development of a time-sampling observational technique for describing children's experiences in preschool settings. The observational technique was developed in an earlier study to assess the impact of experiences in Headstart programs. In this study two model preschool programs with differences in educational philosophies were compared. We were interested in determining the extent to which the observational instrument was able to distinguish unique characteristics of different kinds of preschool programs. A second and related purpose of the study is to describe differences in children's experiences in programs based on different philosophies. Although there has been a proliferation of models for preschool intervention during the past several years, there is as yet no observational research describing the ways in which these programs vary in terms of children's experiences.

Methods Employed: The sample consisted of 48 children in six preschool classrooms, three based on the Montessori 'prepared environment' model and three on the Bank Street College 'child-centered' model. To provide a representative sample of the different classroom experiences of children in the two models, each child was observed for ten observational units of two

minutes duration on each of six days. Our previously developed observational technique was refined for use in this study. The observational scheme describes the following dimensions: child-behavior variables, focusing on the nature of a child's cognitive, perceptual-motor, language, social and affective experiences; teacher-behavior variables, including components of teacher control, cognitive input, affective interaction with children, and evaluative feedback; and structural components of the environment, including the degree of structure of classroom materials and the extent of freedom given the child in choice of activities.

Major Findings: Statistical comparisons of the programs have been completed using analysis of variance. The comparisons show that the scale is differentiating between the programs on predicted dimensions. For example, there is a significantly greater amount of fantasy play and self-expressive behavior of the children in the Bank Street Schools. In the Montessori classrooms there is significantly more time spent on pre-academic activities, such as, reading, writing, quantitative skills and in visual discrimination.

In addition to the univariate analyses performed last year several multivariate analyses were performed this year. These were of two types: between program (multivariate analysis of variance and discriminant analysis) and within program (factor analysis). The between program analyses indicated that the major dimension differentiating the programs was whether or not the child's activities allowed for many or few response possibilities (unstructured vs. structured activities). In addition, the amount of fantasy play the child participated in was an important distinguishing variable. The factor analysis for each program separately gave a somewhat different picture. For each program there were two independent dimensions which accounted for significant portions of the variance, but the structure of these dimensions did not correspond to that obtained by the between program analyses. This is not particularly surprising, but it does point up the fact that the variables necessary to describe a program will depend on the particular purpose of the description.

Significance to Biomedical Research and the Program of the Institute: Controversies about the relative merits of various approaches to preschool education and intervention have existed for several years. A major factor preventing resolution of these controversies is the lack of descriptive data based on direct observation of these different programs. The instrument refined in this project should provide a tool for further observational research. The substantive findings from the project should provide important data about the differing experiences of children in various programs.

Proposed Course of Project: The report from this project will be published along with the reports of the Head Start project.

Honors and Awards: None

Papers Presented at Professional Meetings:

Dr. Robert Klein has organized a symposium "Multivariate Comparison of Two Model Preschool Programs" as part of the 1973 meetings of the International Society for the Study of Behavioral Development in Ann Arbor, Michigan. In addition to chairing the symposium he will give a paper on the results of this project, particularly the multivariate analyses.

Serial No. HD-SB2 (c)

1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: An Observational Study of Father-Infant Interaction

Previous Serial Number: Same

Principal Investigators: Frank A. Pedersen, Ph. D., David Schachter, M.D.

Cooperating Units: Pediatrics & Obstetrical Service, National Naval Medical Center

Man Years:

Total:
Professional:
Other:

Project Description:

Objectives: To obtain descriptive data on the early father-infant relationship in natural settings. A methodological component of the study will compare the types of information obtained from interviews of the fathers with data obtained in direct observation of father-infant interaction. The study will also attempt to determine whether there are mediated paternal effects, i.e., whether the father affects the mother's relationship with the infant. A major goal of the study will be to develop a conceptual model for understanding paternal influences as a part of the early environment.

Methods Employed: The sample will consist of approximately 40 middle-class fathers and their first-born infants (the same sample used in Project HD-SB4(c). At age four weeks the father is interviewed regarding the early postnatal period, his current relationship with the infant, and aspects of the husband-wife relationship which may bear on how each parent relates to the baby. At age five weeks an observation is conducted in the home, usually in the early evening, focusing on the father's interaction with the baby. The observational system is an adaptation of the method described in Project HD-SB4(c). Data from that project on the infant and the mother-infant relationship will be utilized in certain analyses.

Major Findings: In focusing on the earliest periods of the father's contact with the baby, we have attempted to conceptualize the role of the father in terms of three areas: (1) his behavior in direct interaction with the baby; (2) his behavior which is instrumental to the mother, and (3) his emotional support given to the mother during her early adaptation to the baby. We have developed an interview that covers these areas. Inter-rater reliability has been established on variables relevant to these areas, and we have coded approximately 25 interviews. Observations have been completed on these cases as well, although we have not begun formal tabulations of these results. We are impressed with the wide range of differences in fathers in all areas, but it is too early in the data collection process to give more than impressionistic results.

Significance to Biomedical Research and the Program of the Institute: Most of our knowledge of early parental behavior is limited to the mother-infant unit. The apparent assumption that this is a closed system, relatively unaffected by the father, and that early paternal behavior is of no consequence is at least open to question. The observational data of father-infant interaction will be unique in their own right, and it is hoped that this study will contribute to our understanding of the infant's early environment.

Proposed Course of Project: We expect to complete the data collection in this fiscal year. Data analyses will be carried out in the coming year.

Honors and Awards: None

Serial No. HD-SB4(c)
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of Infant Temperamental Characteristics on Mother-Infant Adaptation

Previous Serial Number:

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Stuart Copans, M.D., Michael Duchowny, M.D., Robert Klein, Ph. D., A. Bradley Soule, M.D., Kay Standley, Ph. D., Richard Cain, M.Ed., Myrna Fivel, M.A.

Cooperating Units: Pediatrics and Obstetrical Services of National Naval Medical Center; Childbirth Education Association

Man Years:

Total:	5.75
Professional:	5.50
Other:	0.25

Project Description:

Objectives: To study the effects of infant temperamental characteristics on patterns of maternal care and attitudes towards the infant. Within this overall objective there are a number of specific methodological and substantive questions:

1. Is there a basic consistency between infant characteristics assessed in the neonatal period and infant characteristics at four weeks?
2. What are the relationships between mother-infant adaptation at four weeks and certain prenatal variables: her attitudes and expectations regarding the infant; the husband-wife relationship during pregnancy; the woman's physical and psychological adaptation to the pregnancy experience?

3. To what extent do infant temperamental characteristics influence maternal caretaking patterns and mother-infant adaptation? Does a discrepancy in activity level between mother and infant lead to disturbed adaptation? Is there a relationship between the infant's visual alertness and amount of visual stimulation provided by the mother?

Methods Employed: The sample consists of 40 parents and their first-born infants, approximately 20 boys and 20 girls. Only normal infants, free from complications during pregnancy and delivery have been included in the study.

There are five phases of data collection:

1. Prenatal interview with prospective parents in which basic demographic data are obtained. Both parents are interviewed about their expectations and feelings about the unborn child, the degree of solidarity between husband and wife and general adaptation to pregnancy.
2. Neonatal assessment of infant in newborn nursery. Infant characteristics such as visual alertness, auditory responsiveness, irritability, consolability are evaluated using the Brazelton Neonatal Assessment Scale.
3. Four week assessment of infant using the Brazelton Scale.
4. Four week time-sampling observation of mother-infant interaction in the home.
5. Interview with the mother at four weeks regarding her feelings towards the infant and her perception of infant characteristics.

Major Findings: Data collection has been completed on 35 cases. It is planned to complete data collection by July 1973.

Some preliminary analyses have been carried out on the reliability and clinical usefulness of the Brazelton Scale. To assess the clinical value of the Scale comparisons were made between normal infants and babies born to mothers on methadone maintenance. Significant differences were found at three days between the normal and methadone infants on several dimensions, e.g., sensory responsiveness and irritability, indicating that the scale is sensitive to differences in the newborn period. These findings were reported on a symposium on the Brazelton Neonatal Assessment Scale at the Biennial meetings of the Society for Research in Child Development by Bradley Soule.

Significance to Biomedical Research and the Program of the Institute: Most research on early development has concentrated on environmental influences on the young infant conceptualized in essentially unidirectional terms, i.e., the effects of environmental events on infant development. This model is being replaced by an interactional one in which the child is seen as an active agent who influences the behavior of other people towards him. This investigation should begin to give some understanding of reciprocal interactions between infant and mother.

Proposed Course of Project: Collection of data will be continued during this fiscal year. Analyses of data will be made during FY 1974.

Honors and Awards: None

Publications:

Copans, S. A.: Human prenatal effects: A suggested methodology for future study. Merrill-Palmer Quarterly Journal of Human Development, in press.

Papers Presented at Professional Meetings:

Soule, B., Standley, K., Copans, S. and Davis, M.: Clinical implications of the Brazelton Scale. Paper presented at the Biennial Meetings of the Society for research in Child Development, Philadelphia, Pa., March 1973.

Serial No. HD-SB5
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Development of Measures of Social and Motivational Functions in Young Children

Previous Serial Number: None

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Colleen Rand, Ph. D., Kay Jennings, M.A., Sarah Friedman, M.A., Martha Weiss, Leonie Black

Cooperating Units: Institute for Development of Human Resources, University of Florida; Developmental Research Laboratory, Temple University; Syracuse University Children's Center

Man Years:

Total:	2.0
Professional:	2.0
Other:	

Project Description:

Objectives: Although there are many tests of cognitive functioning for young children, there are few standardized instruments for measuring other important aspects of development. In this study we have worked on the development of measures of social and motivational functions, i.e., persistence and trust, for use with children between six months and three years. This has been a collaborative undertaking with the University of Florida, Syracuse University and Temple University.

Methods Employed: Subjects were white middle-class infants tested within a week of their six and twelve month birthdays and within three weeks of their second and third birthdays.

We have concentrated on developing a series of measures for six and twelve month infants. Measures developed for the two and three year olds at the other centers have also been tried out in our laboratory.

Major Findings: Measures of trust in strangers and persistence with objects have been developed for all age groups. In Bethesda 41 infants have been studied with these measures.

Significance to Biomedical Research and the Program of the Institute: Measures that have been used to evaluate compensatory educational programs or the effects of day care programs on the development of young children have for the most part been limited to cognitive development. The measures developed in this project will provide means of assessing other important aspects of development that may be influenced by these programs.

Proposed Course of Project: Data collection will be completed early in the next fiscal year.

Honors and Awards: None

Serial No. HD-SB6

1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: Factors Associated with the Development of Mother-Infant Attachment During the First Year

Previous Serial Number: None

Principal Investigators: Leon J. Yarrow, Ph. D., Robert Klein, Ph. D.

Other Investigators: Joan Durfee, M.A., Myrna Fivel, M.A., George Morgan, Ph.D.

Cooperating Units: None

Man Years:

Total:	.60
Professional:	.50
Other:	.10

Project Description:

Objectives: Although there have been a number of studies on the development of the distinctive tie between mother and infant during the first years of life, there is as yet little understanding of the factors that influence the development of this unique focused relationship. The major objective of this study is to assess the factors in the mother and infant, and in the early mother-infant interaction which affect the nature of this relationship. The second objective is methodological. The studies that have been done on attachment have used a variety of techniques--interview with the mother, structured situations in the laboratory--and a variety of indices to measure attachment. In this study, we plan to compare data obtained from observations in the home, from an interview with the mother and from observations of structured situations in the laboratory. Underlying this methodological question is the conceptual issue of what are the most appropriate behavioral measures of this relationship.

Methods Employed: The sample will consist of forty mothers and their first born twelve-month-old infants. Some of these infants will be part of the sample first studied prenatally (Project No. HD-SB4(c). The remainder of the sample will have entered the study at six months.

Two home observations of approximately three hours duration are conducted within two weeks of the baby's first birthday. These observations code

selected mother and infant behaviors in a number of critical situations which are comparable to measures that have been used in laboratory studies of attachment behavior. The infant's reactions are coded to such events as the mother entering the room, leaving the room, the appearance of the mother when the infant is crying. The infant's vocalizations, smiles, approach behaviors, cessation of crying are recorded.

Following the completion of the two home observations, the mother and infant are brought to the Social and Behavioral Sciences' playroom for a series of observations.

1. The infant's initial response to the female experimenter/interviewer is observed from behind a one-way mirror. The experimenter approaches the infant in a pre-determined, structured but natural manner.
2. The mother is interviewed, using a semi-structured interview schedule regarding selected aspects of her baby's social functioning in relation to herself, the baby's father, other familiar people, and strangers.
3. During the interview, the infant is provided with toys and his exploratory behavior and social initiative and responses (vis a vis mother and interviewer) are observed from behind the one-way mirror.
4. At the completion of the interview proper, the mother is asked to leave the room and the infant's reaction to being left with the interviewer is recorded.

Major Findings: Methods for observation of critical situations in the home have been developed and pretested. Observer reliability has been established. Data collection has just begun. Five infant-mother home and laboratory observations have been made.

Significance to Biomedical Research and the Program of the Institute: The study of "attachment" is central to understanding the development of affectional relationships. Most of the research on attachment has been carried out in contrived situations of a rather stressful nature. There has been little comparison of reactions of infants in these experimental situations with behavior in natural settings. Also very limited is our knowledge of the contribution of temperamental factors in the infant on the developing mother-infant affectional relationship.

Proposed Course of Project: Data collection will be carried on during the next fiscal year.

Honors and Awards: None

Publications:

Yarrow, L. J.: Attachment and dependency: A developmental perspective. In Gewirtz, J. L. (ed.): Attachment and Dependency. Washington, D.C., V. H. Winston & Sons, Inc., 1972, pp. 81-95.

Serial No. HD-SB7

1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Early Environmental Influences on Cognitive and Personality Development During Infancy: A Replication with a Middle-Class Sample

Previous Serial Number: None

Principal Investigator: Leon J. Yarrow, Ph. D.

Other Investigators: Robert P. Klein, Ph. D., Frank A. Pedersen, Ph. D., Joan Durfee, M.A., Myrna Fivel, M.A., Stuart Copans, M.D., A. Bradley Soule III, M.D.

Cooperating Units: None

Man Years:

Total:	1.75
Professional:	1.50
Other:	.25

Project Description:

Objectives: In a previous study with a sample composed largely of infants and mothers of low socioeconomic status some significant relationships were found between a number of early environmental variables and infant development. These findings had important implications for developmental theory. Because of their theoretical significance, it seemed important to test the generality of these findings to determine whether similar relationships would be found in other environmental contexts with different levels and patterns of stimulation. Therefore, we are replicating the previous study with a middle-class sample.

Methods Employed: The sample will consist of 40 infants, 20 boys and 20 girls and their mothers. It is planned to study at five to six months of age the infants first studied as neonates in the reciprocal interaction project (See Project No. HD-SB4).

Data on the environmental variables are being obtained by time-sampling observations in the home using the mother-infant interaction schedule

developed in the previous study. Measures of infant characteristics are derived from the Bayley Scales of Infant Development and through a situational test designed to measure exploratory behavior and preference for novel stimuli.

Major Findings: Data have been collected on 19 subjects.

Significance to Biomedical Research and the Program of the Institute: All too frequently theoretical derivations in the behavioral sciences are based on a few studies in limited experimental or natural situations in which the sample is limited to one cultural group. If we are to make meaningful generalizations about the significance of specific aspects of early experience for development, it is necessary to examine these relationships in a variety of environmental contexts.

Proposed Course of Project: Data collection will continue during the next fiscal year.

Honors and Awards: None

Serial No. HD-SB8
1. Social and Behavioral Sciences Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Factors Influencing Career Choice in Women

Previous Serial Number: None

Principal Investigators: Kay Standley, Ph. D., A. Bradley Soule, III, M.D.

Cooperating Units: None

Man Years:

Total:	.40
Professional:	.40
Other:	

Project Description:

Objectives: To study facilitators and barriers to women's entry into elite occupations by examining the family backgrounds and early life histories of some women who made innovative career decisions. Another area of major interest is the women's perceptions of sex discrimination in their professions.

Methods Employed: The sample consisted of 71 women in law, medicine, and architecture who for the most part resided in the Washington area. Each was interviewed concerning her family and personal history as well as her vocational decisions and current position.

Major Findings: In the analyses completed to date, it has been possible to describe the population as one emerging from urban, affluent, educationally advantaged and professional backgrounds. Parents seem to have been very involved and invested in the academic achievement of these women. In terms of their current lifestyles, most of the women noted a disturbing discontinuity--in some instances conflict--between their careers and their personal (non-occupational) lives. The women commonly reported "dual personnae" themes--instances in which social expectations of them at work and at home were in difficult contradiction.

Significance to Biomedical Research and the Program of the Institute: There is increasing social and practical concern about the relatively few women in elite occupational categories. Study of the vocational

development of successful women should aid our understanding of the obstacles encountered by women seeking vocational fulfillment.

Proposed Course of Project: The project has been completed.

Honors and Awards: None

Publications:

Soule, A. B. and Standley, K.: Lawyers' perceptions of sex discrimination in their profession. Journal of American Bar Association, in press.

Standley, K. and Soule, A. B.: Women and architecture. Journal of Architectural Education, in press.

Standley, K. and Soule, A. B.: Women in male-dominated professions: Contrasts in their personal and vocational histories. Journal of Vocational Behavior, in press.

Papers Presented at Professional Meetings:

Standley, K. and Soule, A. B.: Women in professions: Historic antecedents and current lifestyles. Presented at the annual meeting of the American Psychological Association, Honolulu, September 1972.

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Reproduction Research Branch

SUMMARY

The mission of the Reproduction Research Branch is the development of scientific data and theory basic to reproductive biology. Research efforts therefore extend from the chemistry of the protein and steroid hormones to physiologic and clinical studies in man. To prosecute these investigations successfully, a variety of tools and skills are necessary: physical chemistry of macromolecules, protein isolation and analysis, tissue and organ culture, ultrastructure, enzymology, radioligand assays of protein and steroid hormones, mathematical analyses of immunoassays and the kinetics of protein hormone binding, characterization of *in vitro* steroid biosynthetic pathways, dynamic analysis of steroid hormone metabolism in man. A continuing interest and involvement in clinical problems are not only desirable but necessary since the results of study in man are the final arbiter of the relevance of results in the laboratory.

There have been several important contributions from those investigators working in the broad area of protein chemistry. Prolactin was isolated from amniotic fluid and a practical method was developed for preparation of the hormone. The identity of serum, urine, and amniotic fluid prolactin was proved by physico-chemical, biologic, and immunologic criteria. To define the molecular parameters of chromaffin granule membrane proteins, a novel system for discontinuous buffer electrophoresis in SDS at neutral pH was devised. Luteinizing hormone-releasing hormone (LHRH) was monoiodinated with ^{125}I with preservation of biologic activity, and methods were found to separate labeled from unlabeled species to obtain a tracer of maximum specific activity. The analysis and synthesis of several membrane-active peptides of the gramicidin and nisin series were reported.

Studies in the mechanism of hormone action have taken several directions. A mathematical theory of drug-receptor interaction has been adapted and extended to apply to current problems and validation is being sought from experimental studies and by computer simulation. X-ray diffraction studies of the chromaffin granule membrane revealed the existence of sub-membrane particles distributed symmetrically and composed of proteins. This is the first step in the analysis of membrane changes accompanying secretion. Solubilization and partial purification of soluble receptors of the testis for LH and HCG were achieved. The receptor had a MW of about 200,000 and retained its high affinity and specificity for HCG. Stimulation of steroidogenesis by HCG occurred without detectable increase in cAMP production suggesting that cAMP may not be an essential intermediate in LH action. Specific receptor sites for angiotensin II were demonstrated in adrenal cortex homogenates, probably on microsomal membrane fraction.

The techniques of the cell biologist have proved useful. A clone of cells has been isolated from a hamster ductus deferens tumor that contains a specific protein receptor for both testosterone and estradiol. Proceeding from last

year's observations of the effect of estrogen on follicular development, it has been shown that porcine granulosa cells contain an estrone receptor.

The inception of embryogenesis on the sea urchin is accompanied by net synthesis of polyadenylic acid, a probable requisite step before translation of preexisting genetic messages. The embryo was shown to contain DNA polymerases capable of using RNA templates for DNA synthesis, thereby providing an alternative mechanism for achieving new informational content. The lysosome of the human term placenta was examined and two populations identified. The first trimester placenta is 6 to 8 times richer in lysosomes than term placenta. Conventional lysosomal enzyme markers are not satisfactory for placental lysosomes.

The gonadotropins remain a major focus of interest. In collaboration with Dr. R. Canfield, Columbia University, sub-units of HCG have been prepared, characterized chemically, immunologically and biologically and distributed to investigators around the world. Specific antisera were prepared for each sub-unit and made available to the National Pituitary Agency for distribution. The sub-units were shown to be free of biologic activity. Free HCGα was found in plasma throughout pregnancy thereby accounting for previously noted discrepancies between biologic and immunologic measurements of HCG. The sub-units of HCG have been measured in tissue, serum, and urine of patients with HCG-secreting tumors and the finding that individual metastases had differing contents of sub-units has been interpreted as evidence that cloning of cancer cells occurs in man.

A new technique for a simple solid-phase radioimmunoassay of steroids has been reported. Measurement of cortisol and estradiol in plasma can be made in two hours. A method was developed for measuring free estradiol in urine since it is the best measure of total estrogenic load. The filtration of estradiol by the kidney was estimated using measurements of non-protein bound plasma estradiol.

In the clinical area, the time devoted previously to development of methods has facilitated several new studies. A group of women with secondary amenorrhea and weight loss was identified in whom the mechanism of the amenorrhea could be attributed to hypothalamic dysfunction on the basis of testing of pituitary and hypothalamic function. The increased sensitivity of an improved human prolactin assay facilitated investigation of the hypothalamic-pituitary unit. High plasma prolactin concentrations were noted in some patients with pituitary tumors but without lactation.

The male infertility clinic continues to serve to identify "experiments of nature" that illuminate testicular regulatory mechanisms. Serum FSH is high when germ cells are absent. It has been difficult to decide whether the germ cell or Sertoli cell is responsible for regulation of FSH levels. The finding in three men that a normal sperm count with death of all sperm associated with high FSH is strong evidence that the Sertoli cell is the regulator of FSH secretion. A clinical trial has been initiated for the use of high doses of FSH in various types of male infertility.

The clinical studies have been complemented by related work in the rat. Vitamin

A deficiency has been used to achieve reversible germ cell depletion. The FSH did not rise again implicating the Sertoli cell as the source of FSH regulatory control. A study of enzyme markers in the testis suggests that γ-glutamyl-transpeptidase may be a marker for the mature Sertoli cell.

Research utilizing the primate colony has been carried out by collaborations among many members of the Branch. Pregnancy in the rhesus monkey is not characterized by continued excretion of chorionic gonadotropins as in man. A radioimmunoassay for monkey CG was developed and is useful in purification and clinical studies. The antigenicity of gonadotropins of primates was examined. Antisera to hCGβ recognized common antigenic determinants in man, gorilla, and chimpanzee but not in the rhesus monkey. Electron microscopy of the rhesus corpus luteum throughout pregnancy was performed and the morphologic features are consistent with continuing steroidogenesis. Ovariectomy in the rhesus after day 24 caused no abnormalities in sex steroid concentrations in the blood and peak estradiol levels were attained at day 35 as in the normal pregnant monkey. Thus, as in man, corpus luteum function during pregnancy is essential only during the early weeks of pregnancy.

The Branch will again undergo change. Dr. W. Tullner is retiring after many years of productive research and leadership. Dr. Gary Hodgen will be Acting Head, Endocrinology Section. Dr. Peter O. Kohler is moving to Baylor University. His contributions in the laboratory and ward will be difficult to replace. Dr. D. L. Loriaux will serve as Acting Head, Clinical Service. Dr. Kevin Catt will become Head of a new section, Hormonal Regulation, and expand his efforts in several aspects of regulatory mechanisms. The Section of Molecular Structure, headed by Dr. E. Gross has joined the Branch.

Our labors this year have again been aided greatly by visiting scientists, fellows, and guest workers. These scientists and our Clinical Associates have been a continued source of stimulation. The collaborative efforts within the Branch and with other scientists in Bethesda and elsewhere have strengthened the research and multiplied our effectiveness. Finally, the support of the Institute for the use of service contracts has enabled us to shift some of the burden of routine work and has thereby freed our staff for more creative research.

Serial No. HD-RPL1
1. Reproduction Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biology of Reproduction: Clinical and Experimental

Principal Investigator: Dr. Mortimer B. Lipsett

Other Investigators: Dr. G. T. Ross, Dr. W. W. Tullner, Dr. P. O. Kohler, Dr. R. J. Sherins, Dr. D. Rodbard, Dr. A. Chrambach, Dr. H. Pollard, Dr. K. Catt, Dr. M. D. Catt, Dr. B. Gulyas, Dr. W. Nixon, Dr. G. Hodgen, Dr. D. Loriaux, Dr. C. Cargille, Dr. E. Gross, Dr. H. Chen, Dr. R. Becker, Dr. P. Krueger, Dr. B. Nisula, Dr. R. Mecklenburg, Dr. L. Corash, Dr. W. Gerber, Dr. P. Fredlund, Dr. J. Hansen, Dr. R. Vigersky, Dr. S. M. Harman, Mr. P. E. Rayford, Mr. D. Ryan, Mr. G. Coffman, Mrs. M. Wyckoff, Mr. C. Turner, Mr. D. Barber, Mr. R. Reid, Mr. J. Brice, Mrs. M. Collins, Miss J. Brown, Mr. J. Morell.

Cooperating Units: Veterans Administration Hospital, Palo Alto; Department of Applied Mathematics, Harvard University, Boston; Department of Anatomy, Harvard University, Boston; Univ. of Paris School of Medicine, Paris; Max Planck Inst., Gottingen; Columbia Univ. School of Medicine, New York; Georgetown Univ. School of Medicine, Washington, D.C.; Emory Univ. School of Medicine, Atlanta; M. D. Anderson Inst., Houston; Physical Sciences Laboratory, DCRT; Laboratory of Biochemistry and Metabolism, NIAMD; Section of Mineral Metabolism, NIAMD: Laboratory of Experimental Pathology, NIAMD; Laboratory of Cell Biology, NCI; Laboratory of Biomedical Sciences, NICHD; Department of Obstetrics & Gynecology, National Naval Medical Center, Bethesda; Laboratory of Clinical Science, NIMH; Mass. Institute of Technology, Cambridge.

Man Years:

Total: 62
Professional: 38
Other: 24

Project Description:

Objectives:

1. To understand molecular mechanisms of hormone action;
2. To define pituitary and gonadal secretion in health and disease;
3. To explore the processes involved in maintenance of blood and tissue hormone levels;
4. To develop methods for studying the interaction of the seminiferous tubule and the pituitary hypothalamic unit;
5. To describe functionally the biology of germ cell maturation in the gonads.

Methods Employed:

A great variety of techniques are employed since the research encompasses such widely divergent areas as mathematical biology, protein hormone chemistry and physiology, tissue culture, steroid biochemistry, morphology, and clinical investigation. New methods that have been either developed or adapted during the year are:

A new more efficient solid-phase method for peptide amide synthesis;

The use of fluorescent techniques to identify lysosomes in tissue. This facilitates the preparation of lysosomes;

New chemical non-enzymatic degradative techniques for structural elucidation of membrane-active peptides with unusual structural features;

The use of isoelectric focusing on sucrose columns as a preparative technique for protein purification;

Adaptation and validation of a method for rapid extraction of gonadotropins from urine for quantitation by immunoassay;

Development of a simple fast radioligand assay for steroid hormones;

An improved method for radioimmunoassay of Angiotensin II;

The calculation of affinity constants and binding capacity with routine testing of four alternative models.

Major Findings:

I. Protein Chemistry

Since amniotic fluid is a good source of prolactin, it was necessary to show that this prolactin was the same as pituitary and serum prolactin. This identity was proved by physico-chemical criteria, biologic assay, and immunologic cross-reactivity. The high isoelectric point of prolactin suggested that efficient separation from isohormones of growth hormone would be achieved

by isoelectric focusing. Small amounts of homogeneous prolactin were prepared by gel filtration, isoelectric focusing and gel electrophoresis. From this experience, the procedures are now being adapted for preparative work.

Two years ago it was reported that plasmin digestion of growth hormone yielded a growth hormone with a ten-fold increase in specific activity. Preparative methods have now been devised for isolation of milligram amounts of this substance.

The methodology of separating any charged species from any other under optimal conditions and with "quantitative" physical characterization of the species nas been published, bringing to completion a project of 10 years. The papers cover every major aspect of fractionation on polyacrylamide gel. The comprehensive theory and discussion of the various aspects of fractionation on polyacrylamide contributes a strategy to fractionation that is at the same time universal and flexible. At each step of this strategy, we have provided apparatus, procedures and computer programs for the rapid and error-free evaluation of the data and their statistical limits. The programs take the measured electrophoretic mobility, or relative mobility, of a band and (1) convert it to measures of molecular size and net charge with their confidence limits; (2) calculate the gel concentration for optimal resolution between any set of 2 components; (3) define identity or non-identity between any 2 components; (4) simulate the time course of preparative elution fractionation on PAGE and indicate the time, for any gel volume, required for the quantitative separation of any component from any other. This set of computer programs has been made available to the public via commercial time sharing and by mailing out of tapes.

Another program's package, developed by use of T.M. Jovin's theory and program for the analysis and generation of multiphasic buffer systems, extends the applicability of gel electrophoresis and "stacking" to the entire pH range.

Apparatus and procedures have been developed for analytical PAGE and IFPA, transverse and longitudinal gel sectioning, autoradiography of gel slices, formation of pore gradient gels, preparative PAGE and IFPA, automatic elution flow rate deceleration, eluate concentration, eluent reservoirs of adjustable level, determination of polymerization efficiency, determination of boundary displacement, gel slab electrophoresis in discontinuous buffer systems, and numerous other minor items. We are in the process of developing a procedure for preparation and purification of iodinated proteins which will combine in one purification of the iodinated protein. This should also provide vastly improved radiation safety compared with present techniques.

The importance of this body of theory and experience was recognized at the NIH by a request for a special course for NIH personnel. Drs. Chrambach and Rodbard taught a two-week course to 20 NIH sicentists.

New methods for calculating affinity constants ("K") and binding capacities, ("q") have been developed. In particular, the programs now routinely test four alternative models and then select the model with the minimum number of

parameters required to give a satisfactory fit. Computations which formerly required a full day's work with a complicated "custom-fit" program are now done routinely in a few minutes.

The vast majority of "K" and "q" values in the literature are calculated incorrectly (no weighting, correlation of errors in X and Y, incorrect models, improper "curve peeling"). A major attempt to address this problem was the writing of several review articles and chapters on this subject. The theory of protein-ligand binding will be useful in a variety of studies concerned with the concentration of "free" (unbound) steroids in plasma.

A mathematical theory to describe the transient state of isoelectric focusing (pH gradient electrophoresis) was derived. This should be useful in connection with experimental studies, especially those dealing with determination of physical-chemical constants (e.g., diffusion coefficients) from PAGE and isoelectric focusing.

In order to prepare labeled LHRH for clinical studies, iodination was performed using the lacto e oxidase method. Under appropriate conditions, labeled LHRH was separated from unlabeled material, thus yielding a tracer of maximum specific activity. It was shown to be monoiodinated and to retain full biologic activity.

Proposed course of project: The preparation of sufficient amounts of pure prolactin and growth hormone isohormones for structural studies will be undertaken.

II. Mechanism of Hormone Action

The mathematical theory for hormone-receptor and drug-receptor interaction, as developed by the pharmacologists, has been reviewed, revised, and extended to apply to current problems in mechanism of hormone action. This work is based on, and represents an extension of previous studies from this laboratory on protein-ligand interactions, antigen-antibody reactions, and the theory of radioimmunoassays.

The paradoxical dissociation of "binding" curves and "response" curves in in vitro systems may be due to allosteric effects, heterogeneity of binding sites, or a quantal mechanism whereby individual cells in the target tissue respond in a quantal fashion when a threshold number of receptor sites are filled. Current studies are aimed at distinguishing among these possibilities, and involve both experimental design, computer simulation studies, and data analysis.

A method was developed for treating membranes of bovine chromaffin granules in such a manner as to orient them on a planar surface. This allowed X-ray diffraction studies to be performed and structural information to be obtained. The specific result was that the membrane is chemically asymmetric and that objects of approximately 70 Å in diameter are distributed in the plane of the membrane in a symmetric fashion. The symmetry may be, in part, hexagonal.

Electron microscopy of the membranes revealed that the product was vesicular,

as was the original organelle. This was confirmed by both thin section and negative stain methods. Freeze-fracture electron microscopy confirmed: (1) existence of submembrane particles, approximately 70Å in diameter; (2) asymmetric distribution of the particle with regard to membrane surfaces; and (3) ordered arrays of particles in the membrane plane, showing 5-fold axes and surrounded by 6-fold axes.

Protein chemistry studies on these membranes revealed that there were at least 8 major species by disc gel electrophoresis in SDS. All had molecular weights of less than 65,000. A novel system for doing discontinuous buffer electrophoresis of membrane protein in SDS at neutral pH was developed. This system has been used to define the molecular parameters of the membrane protein more precisely and to make a tentative identification of dopamine-β-hydroxylase, relative to a sample of the purified enzyme. The membrane was also found to be up to 20% cytochrome b_{562} and cytochrome b_{562} reductase, suggesting that electron transport is an important membrane function.

Quantitative lipid analysis has corroborated our measurements of hydrated density from sucrose gradients (approximately 1.12 gcm^{-3}) and yielded an estimate that the membrane is 40-45% protein. Thin-layer chromatography has revealed the presence of common phospholipids, but lysolecithin is present in only small quantity. Previously it had been reported that the membrane was unusual in that up to 20% of the phospholipids were lysolecithin.

Release of cyclic AMP and testosterone by the incubated rat testis has been determined during further studies on gonadotropin action in vitro. Stimulation of steroidogenesis by low gonadotropin concentrations ($<10^{-11}$M hCG) has been shown to occur without an accompanying rise in cyclic AMP production, suggesting that cyclic AMP may not be an essential intermediate in the mode of action of LH on the Leydig cell. This observation has also been made in isolated Leydig cells prepared by collagenase dispersion of the rat testis. Quantitative binding studies of dispersed interstitial cells have shown that the number of LH/hCG receptors per cell is about 50,000.

A requirement for calcium in gonadotropin action has also been demonstrated in the rat testis. Removal of Ca+ by EGTA abolishes the release of cyclic AMP and testosterone in response to hCG, and also blocks the testosterone response to dibutyryl cyclic AMP. Binding of gonadotropin is not affected by such changes in calcium concentration, indicating that the requirement for calcium is at the level of receptor coupling to adenylate cyclase and also at later stages of steroidogenesis.

Gonadotropin receptors for LH and hCG were extracted from cell fractions of the testis and ovary with the non-ionic detergent, Triton X-100. The solubilized receptors remained in solution after centrifugation at 360,000 g for 2 hrs and comprised more than 90% of the original particulate binding sites.

For binding studies of the soluble receptors with ^{125}I-labeled hCG, separation of bound and free tracer was accomplished by precipitation of the bound complex with polyethylene glycol. This assay system has been extensively used to carry out binding and kinetic studies, as well as for monitoring

receptor activity during physico-chemical studies. The binding was temperature dependent, with higher rate of uptake at 37°C but lower binding capacity due to receptor degradation at higher temperature. The soluble receptor retained hormonal specificity for LH and hCG and displayed high affinity for hCG at 0° and 24° (Ka = 0.5-1 x $10^{10}M^{-1}$) and low capacity 10^{-12}mole/gm. The association rate constant was 6.1 x 10^{5} M^{-1} min^{-1} at 4°C. The dissociation occurred extremely slowly with a first order dissociation rate constant of 1.2 x $10^{-4}min^{-1}$. Comparison between testicular and ovarian binding data showed striking similarities. Combining data obtained from gel filtration studies on Sepharose 6B columns and from sucrose gradient centrifugation on the free receptor and receptor-hormone complex the size and shape of the molecules were calculated:

Hydrodynamic radius 64Å (for both forms)

Sedimentation constant

free receptor	6.5S
hormone-receptor complex	7.5S

Molecular weight

free receptor	194,000
hormone receptor complex	224,000

The axial ratios (prolate) of the two forms were 12 and 10.2 respectively. The properties of the hCG/LH gonadotropin receptors were consistent with those of a highly asymmetric molecule predominantly protein in nature, with relatively high conformational stability.

Specific receptor sites for angiotensin II were demonstrated in the bovine adrenal cortex by binding studies with ^{3}H- and ^{125}I-angiotensin II. The receptor affinity for angiotensin II is higher than that for angiotensin I, and the binding sites in adrenal homogenates are situated in a microsomal membrane fraction with enzymic characteristics of cell membrane. Fragments analogues, and antagonists of angiotensin II have binding-inhibition potencies which are proportional to their biological activities in vivo.

Proposed course of project - It is planned to develop isomorphous derivatives of the membrane in which electron or X-ray dense probes are attached to specific enzymes in the membrane in order to locate them geometrically. These include a ferritin-anti-dopamine-β-hydroxylase reagent suitable for both X-ray and electron microscopic analysis and a ferritin-SH reagent that might allow us to localize the -SH group on the active transport ATPase. These studies would tie in with functional studies on the chemistry of the transport process for catecholamines, and on the mechanism of release of the neurotransmitter from the cell.

Purification of gonadotropin receptors will be continued to obtain enough protein for structure-function studies. Stimulus-secretion coupling in testis and zona glomerulosa of the adrenal cortex will be a continuing focus of work. Angiotensin II receptors will be characterized and studied.

III. Cell Biology

The immunological studies with trophoblast have been extended. Previous work had indicated that lymphocytes from a variety of donors (normal people, pregnant women, women with moles or choriocarcinoma) did not recognize the JEG lines of choriocarcinoma in culture as foreign. This had suggested that histocompatibility antigens were masked or absent. However, another cultured cell line of choriocarcinoma cells, recently isolated in our laboratory, is not recognized as foreign by lymphocytes from some normal people. This suggests that the reason the placenta is not rejected during normal pregnancy and the reason choriocarcinoma spreads so vigorously is not an inability of the cell to express surface antigens.

Of some interest are the findings that administration of antisera to hCG to hamsters bearing human choriocarcinoma results in significant reduction in tumor growth as well as inhibition of the peripheral manifestation of the tumor's hormonal activity.

A clone of ductus deferens tumor cells was established. These cells show a specific uptake of testosterone and estradiol. The specific binding material has been isolated from cytosol and is presently being characterized. Testosterone appears to increase the growth rate of these cells. This cell line will provide an in vitro model for sex steroid action. Since estradiol stimulates the growth of the granulosa cell, the steroid binding properties of porcine granulosa cells in culture were examined. These cells specifically bind estrone and estradiol, and the binding characteristics differ from those of uterine cytosol receptor. However, the binding material appears to be very fragile and a satisfactory preparation from cytosol is not yet available.

Proposed course of project - Since the senior investigator is leaving, it seems likely that most of this work will move with him.

IV. Gonadotropins

In a continuing collaboration with Dr. R. Canfield and workers in New York, immunologic and biologic assays have been used to characterize preparations of human chorionic gonadotropin during purification and production of subunits of human chorionic gonadotropin. Methods have been developed for large scale production of a highly purified reagent for use in structure-function studies. In addition to studies conducted jointly between the two laboratories, these substances have been made available to a number of other investigators around the world. Results of recently completed studies justify the following conclusions: (1) Highly purified alpha and beta subunits have been shown to be free of biologic activity in vivo and in vitro; (2) Except for differences in carbohydrate content, the alpha subunits of hCG and hLH have identical, primary structures; (3) A structural basis has been established for antigenic differences in hLH and hCG. The hCG beta subunit contains about 30 additional aminoacids added onto the C terminal subunit of beta unit of hLH. This terminal portion of hCG beta subunit has been isolated and studies of its antigenic behavior are in progress; (4) In collaborative studies with Dr. Harold Edelboch, NIAMD, it has been shown that subunits of hCG do not bind

ANS, whereas native hormone does. The extent of ANS binding, and thus of recombination, can be monitored by changes in fluorescence of aqueous solutions containing the dye and the subunits so that the kinetics of the recombination can be monitored. Preliminary studies indicate that this method is useful for following the course of reactions during hybridization of complementary subunits of glycoprotein hormones from different species.

Placental tissue was obtained from elective abortions performed at several different hospitals in the area. The tissue was extracted and gel filtered. The eluates were radioimmunoassayed for hCG, hCGα and hCGβ. In the first 6-10 weeks of gestation, three different physical forms of hCGα and two different physical forms of hCGβ were evident. The additional forms of hCG subunits found had a higher apparent molecular weight than did highly purified native subunits. These previously unrecognized forms of subunits could represent intracellular subunits released before final processing. After the 10th week of gestation, the amount of hCGα exceeded hCG by several fold. Since the subunit is devoid of biologic activity, its presence could account for the lower biologic to immunologic activity ratio noted after the first trimester of pregnancy.

Plasma, urine and tumor tissue extracts were studied for altered forms of hCG and its subunits. All subjects studied with nongestational hCG secreting tumors were found to have unbalanced synthesis, secretion or excretion of hCG and its subunits. Interestingly, hCG subunits were not detectable in plasma or urine of patients with molar pregnancies.

100 ml of an hCGβ antiserum was given to the National Pituitary Agency for distribution to interested, qualified investigators. The antisera can be used for early diagnosis of pregnancy, following therapeutic course of patients with hCG secreting tumors and as a screening assay tumor marker since several different neoplasms have been shown to ectopically secrete hCG.

The structure-function inter-relationships of the gonadotropins and various human thyroid stimulators were investigated. Results of these studies support the current concept of an alpha subunit structure of the gonadotropins similar to that of the pituitary thyrotropin but dissimilar to that of the thyroid stimulating activity extracted from placenta. In contrast to the thyroid stimulator extracted from placenta, the thyroid stimulator which circulates during pregnancy is similar in structure to hCG. Immunologic and biologic evidence suggests that a thyroid stimulator distinct from hTSH and LATS circulates in the blood of some men with testicular tumors. This potential tumor marker may be similar to the thyroid stimulator of pregnancy.

Proposed Course of Project - Collaborative studies with the laboratories of Darrell Ward at Houston and Robert Canfield at Columbia are projected for next year. It is proposed to examine the antigenic and biologic properties of hybrids of fragments such as tryptic peptides of alpha subunits with beta subunits of hCG and ovine LH in attempts to define minimal amino acid sequences required for biologic activities.

The "big" subunits found in placental tissue will be characterized and studied

to determine whether hCG is synthesized initially in a "big" form as two other polypeptide hormones appear to be.

V. Clinical Studies

Applications of immunologic and biologic assays for gonadotropins to the study of physiology of human reproduction have continued. Drs. Reiter and Kulin have shown that immunoreactive gonadotropins can be satisfactorily recovered from human urine by precipitation with acetone. This method represents a significant advance over the kaolin acetone method presently used for extracting biologically active gonadotropins. The acetone method is currently being used to compare patterns of urinary gonadotropin excretion in first morning voidings and 24-hour urine collections from normal women and from prepubertal boys and girls.

Approximately 40 new patients with menstrual aberrations were studied over the past year. Testing with LH-RH was added to our protocol to better evaluate pituitary-hypothalamic function in these patients. A new sub-group was identified using this approach. A significant population of patients with secondary amenorrhea presented with a history of voluntary weight loss accompanying cessation of spontaneous menses. None of this group of patients had a clinical diagnosis of anorexia nervosa. As a group, they (1) had low to barely detectable urinary and plasma gonadotropins; (2) failed to respond to clomiphene in terms of a rise of plasma gonadotropin levels, and (3) all responded to LRF with a significant rise of plasma FSH and LH implying that hypothalamic dysfunction was the basis for their clinical syndrome.

In women not responding to clomiphene and who did not have primary ovarian disease, ovulation was induced with sequential parenteral menopausal gonadotropin and hCG. This form of therapy was carried out only in those women interested in conception. Plasma and urine were obtained at monthly intervals from those women who conceived. The plasma and urine were subjected to Sephadex G-100 chromatography and each eluate assayed for hCG, hCGα and hCGβ in homologous radioimmunoassays. In short, free hCGα was found in plasma throughout pregnancy.

The sensitivity of a heterologous system for measurement of human prolactin has been improved by the use of ^{125}I labeled human prolactin. The new assay is precise and sufficiently sensitive for measurement of basal levels of the hormone in serum from normal men and women. This assay has been coupled with pharmacological methods for perturbing pituitary prolactin secretion in diagnostic evaluation of patients suspected of having functional disorders of the hypothalamic-pituitary unit. It has been shown that serum prolactin concentrations are high in most patients having pituitary tumors with or without associated galactorrhea. In addition to these clinical studies, this assay was used to establish the similarity of physical properties of prolactin in human amniotic fluid and pituitary extracts.

The extensive use of radioimmunoassays in clinical investigation has required that data processing be handled efficiently. A new, improved computer program for RIA data processing has been developed and is being distributed by the

National Technical Information Service (magnetic tapes and listings). More than 200 copies of the program have been distributed. Earlier versions of the program have been adapted to virtually all desk-top calculators and minicomputers, and many manufacturers and distributors of RIA reagents have adopted the "logit-log" method, so that it is probably the must widely used of any method developed to date.

The new program provides:

(1) Estimation of K values even for non-linear Scatchard plot;
(2) Testing of linearity, testing of parallelism, and combined potency estimates;
(3) An optimization program, advising on changes needed to make subsequent assays more sensitive;
(4) New input-output options; and
(5) New detailed statistical analyses, providing more efficient utilization of data.

Assays for plasma renin activity (PRA), renin concentration (PRC) and renin substrate (PRS), employing radioimmunoassay of Angiotensin I, have been established and validated, and an improved method for radioimmunoassay of circulating angiotensin II has been developed. These assays are currently being applied to studies in various forms of hypertension, and during oral contraceptive therapy and pregnancy. The most significant result of the clinical studies has been the demonstration that "low renin" hypertension does not protect against heart attacks and strokes as claimed by Laragh. In a large series of hypertension patients (studied in collaboration with the Hypertension Unit of the D. C. General Hospital), those with low renin hypertension were found to have the same incidence of complications as those with normal or high renin levels. This finding has important implications for the management and prognosis of patients with 'low renin' essential hypertension.

The male infertility clinic is providing a major clinical resource for the Washington area. The significance of a low sperm count is being defined and men with consistent oligospermia (total sperm count, 25 million) are able to impregnate their wives. Extensive study has revealed the large variability of the sperm count and emphasizes the necessity for long control periods in order to assess attempts at therapy.

Plasma FSH levels are elevated in azoospermia and, in general, are inversely correlated with the sperm count. Administration of hCG suppresses FSH to very low levels yet spermatogenesis is unchanged. This suggests, in accord with data in other species, that FSH may not be required for maintenance of spermatogenesis.

During attempts at therapy with hCG, many men developed gynecomastia. Since plasma prolactin did not increase and estradiol concentration doubled, it is now appropriate to attribute this gynecomastia to the effect of estrogen.

To facilitate measurement of steroid hormones in clinical studies, a technique of co-polymerization of antisera with polyacrylamide has been developed which renders the separation of bound from free, a time independent process, and

thereby simplifies the method. The technique has adequate sensitivity to measure 4 picograms of estradiol in an unchromatographed extract of human plasma. It is currently being used in the routine measurement of plasma and urinary cortisol and estradiol.

A method has been developed for the measurement of free estradiol in human urine. Urinary free estradiol correlates directly with plasma estradiol through the menstrual cycle. To complement these data, the plasma binding of estradiol at 37° was examined and the association constants of estradiol with TeBG and albumin were measured. From these data and estimation of TeBG binding capacity, we estimate that most of the estradiol filterd at the glomerulus is not reabsorbed.

Because of obvious abnormalities of gonadotropin secretion in women with anorexia nervosa, hypothalamic function in these patients was studied. Patients with anorexia nervosa were unable to maintain core body temperature in a cold environment. Two people, equally cachectic but without anorexia nervosa, could maintain body temperature. The defect persisted in one patient who had recovered from anorexia nervosa. Patients with anorexia nervosa had abnormal responses to a hot environment. Four of five patients had partial diabetes insipidus. Response to LHRH was normal in all but one patient. This patient was taking estrogen and thyroxine, both of which are said to block LHRH activity. Other parameters of anterior pituitary function were normal. These novel findings are interpreted as indicating disordered hypothalamic function in anorexia nervosa.

Moniodinated LHRH was infused into normal volunteers. The initial $t_{1/2}$ was estimated at between 3 and 4 min. The distribution volume approximated plasma volume. Similar data were obtained in the rat.

Projected Course of Clinical Studies - The clinical study of men and women with problems of infertility will be continued since this population of patients is available for analysis of particular endocrine abnormalities as well as for new therapeutic interventions. The mathematical programs for radioimmunoassay will be improved by use of a four parameter logistic model that will provide improved curve fitting and a new "generation" of RIA programs. Hypothalamic disease will be explored extensively by testing with LH-RH and thyrotropin-releasing hormone.

Proposed Course for the Branch

The general area of hormone action will be expanded with the creation of a section headed by Dr. K. J. Catt. Progress achieved in purification of the hCG receptor has been rapid and the stability of this receptor augurs well for future work. The development of techniques for studying protein structure of membranes will permit new meaningful exploration of membrane response to polypeptide hormones.

The need for more biology in the Branch remains. The departure of Dr. P. O. Kohler removes one of our productive biologists. Studies of follicular development and fertilization should constitue an important field of research in the Branch. When positions become available, it is planned to develop such a Section.

Serial No. HD-RPL2
1. Reproduction Research Branch
2. Section of Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Physiology of Testis

Principal Investigator: Dr. William W. Tullner

Other Investigators: Dr. Gary D. Hodgen and Dr. Philip M. Krueger

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives:

1. To develop methods for measuring function of specific tubular cells;
2. To understand the regulation of FSH secretion.

Major Findings:

Having characterized the pattern of three enzymic markers during the initiation of spermatogenesis in rats, we used these testis specific markers to monitor selective germinal cell depletion in vitamin A deficient rats. In 130 day old vitamin A deficient rats, lactic dehydrogenase (LDH) was 62% of controls and LDH-X isozymes were undetectable, indicating the absence of pachytene spermatocytes, spermatids, and spermatozoa. Similarly, loss of these advanced germinal cells was marked by reduced sorbitol dehydrogenase levels to 19% of controls. These findings were confirmed histologically. Despite the virtual absence of germinal cells, γ-glutamyl-transpeptidase (GTP) remained near control levels and Sertoli cells were histologically normal. These findings suggest that the vitamin A deficient rat is a useful model for accomplishing selective depletion of the germinal epithelium and supports earlier data suggesting that GTP may be an enzyme marker for Sertoli cells.

Serum FSH, LH and testosterone levels in adult rats were measured after artificially induced unilateral and bilateral cryptorchidism and unilateral and bilateral castration. The data do not support the view that cryptorchidism is an acceptable model system to test the suppressive effects of an FSH inhibitory substance, said to be released from the seminiferous tubule, since bilateral cryptorchidism resulted in elevations of both FSH and LH. Further,

neither unilateral nor bilateral cryptorchidism had any significant influence on testosterone levels. Removal of the epididymis did not affect LH and FSH concentrations.

A vitamin A deficient colony of male rats has been established because it provides a model to study the reversible depletion of testicular germ cells. Earlier studies in humans demonstrated a selective increase in FSH concentration when germinal aplasia occurred as a consequence of chemotherapy; which suggested that the seminiferous tubule produced a factor capable of inhibiting FSH secretion. In this rodent model, depletion of germ cells occurs down to the spermatogonial level, and yet no changes in plasma FSH have been demonstrated. This strongly suggests that release of the "FSH inhibitor" from the tubule likely occurs at the Sertoli cell and/or low spermatogonial site. This animal model would appear to be extremely valuable for further understanding of the hormonal regulation of spermatogenesis.

Proposed Course of the Project:

Emphasis will be placed in two areas. The exploitation of the vitamin A deficient rat system will be continued to identify the cell involved in FSH regulation. A model system will be devised for use in attempts to isolate tubular FSH-regulating factor.

Serial No. HD-RPL3
1. Reproduction Research Branch
2. Section of Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Fertilization and Embryogenesis

Principal Investigator: Dr. William W. Tullner

Other Investigators: Dr. Bela J. Gulyas and Dr. Donald W. Slater

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives:

1. To describe the ultrastructural features of early embryogenesis;
2. To define the processes involved in processing and controlling of information at the molecular level during early embryogenesis.

Major Findings:

Earlier observations on the contractile ring and the mid-body in dividing rabbit zygotes were further pursued. In late anaphase of karyokinesis a shallow indentation appears in the vitelline membrane in a plane perpendicular to the spindle. Microfilaments form the contractile ring in the shallow indentation. Throughout furrow formation these 50 to 80 A filaments underlie the broad leading edge of the cleavage furrow. Also in late anaphase stem-bodies can be identified centrally in the egg. Each stem-body consists of several microtubules in an electron dense matrix. As division progresses the stem-bodies fuse into a mid-body which at several points fuses with the contractile ring. Primitive intermediate junctions develop between the resulting two blastomeres.

During the course of examining more than 500 rabbit zygotes, which were obtained from normally mated females, approximately 1.1% of them were found to be spontaneously polyspermic. The presence of supernumerary (polyspermic) sperm with varying degrees of transformation into pronuclei, in zygotes of similar age, suggests that entry of the supernumerary sperm can occur at various times after fertilization. The early stages of transformation into pronucleus slightly diverge from that of the normal fertilization. Unlike normal fertilization, the nuclear envelope is formed around the condensed

supernumerary sperm head. Decondensation of the chromatin occurs in the confines of the nuclear envelope, rather than free in the cytoplasm. In nearly all of the polyspermic zygotes, the male and female pronuclei were in one hemisphere of the zygote and the supernumerary male nucleus was located peripherally in the opposite hemisphere. The dislocation of the pronuclei is an abnormal feature since in the normal zygotes the male and female pronuclei migrate to the center of the zygote and remain there until first cleavage.

Unfertilized sea urchin eggs and embryos have been shown to contain two classes of DNA polymerases functionally distinguishable via their potential to employ synthetic DNA.RNA hybrids as well as RNA.RNA duplexes as templates for DNA synthesis. This RNA-mediated DNA synthesis is dependent on the addition of the pertinent polymerases, templates and cofactors and results in an asymmetrically transcribed product the molecular makeup of which is dictated by the RNA moiety of the template employed. Although partially purified polymerase preparations obtained from either unfertilized eggs or embryos contain a similar spectrum of DNA- and RNA-instructed potential polymerases, the divalent cation requirements and thermal labilities of the RNA-instructed holopolymerases are not identical. Examination of the respective polymerase saturation levels and competition experiments between appropriate templates further suggests that both the egg and embryo possess polymerases with differing template affinities.

Molecular hybridization between ^{3}H-poly(U) and unlabeled RNA from unfertilized eggs and embryos has been employed to demonstrate that the inception of embryogenesis is accompanied by a greater than two-fold net synthesis of polyadenylic acid [poly(A)], a species of RNA known to be a proprietary property of metazoan genetic messages. This synthesis was shown to be immune to any tandem transcriptive requirement but significantly stimulated by the inhibition of protein synthesis. Our findings indicate that the controlled adenylation of pre-existing genetic messages present in the ovum is modulated by a post-fertilization translational event other than the synthesis of the pertinent polymerase(s) *per se*. These results thereby represent the first documentation of a fertilization elicited net synthesis of an RNA species and, equally important, suggest that such synthesis is not only under negative genetic control, but that the RNA moiety in question is of a regulatory rather than codogenic nature.

As the vigorous increment in protein synthesis known to follow fertilization precedes the above-noted net synthesis of poly(A) by approximately 60 minutes, the biology of the system,coupled with the procedures employed, permitted a direct comparison of the disposition and characteristics of the preadenylated maternal RNA with those genetic messages adenylated in response to fertilization. The results obtained establish that preadenylated latent genetic messages of maternal origin are predominantly located in the ovum's subribosomal fraction and that fertilization elicits a rapid transposition of these transcripts into the zygote's ribosomal fraction. Thus the observed reallocation of adenylated RNA parallels the increase in protein synthesis which occurs after fertilization and thereby supports the interpretation that the adenylated RNA present in the unfertilized egg primarily represents anabolic intermediates rather than catabolic products of oogenic metabolism.

Proposed Course of the Project:

The molecular biology component will be transferred to the Gerontology Research Center. At the ultrastructural level, the mode of formation of the two types of nucleoli in rabbit embryos will be examined. Further studies on the genesis of the annulate lamellae in rabbit zygotes are planned.

Serial No. HD-RPL4
1. Reproduction Research Branch
2. Section of Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Use of the Rhesus Monkey in Studies of Pregnancy

Principal Investigator: Dr. William W. Tullner

Other Investigators: Dr. Gary D. Hodgen and Dr. Wilbert E. Nixon

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives:

1. To develop methods for measurement of pituitary and placental hormones;
2. To evaluate the function of the corpus luteum;
3. To describe and define the sources of steroid hormones during pregnancy.

Major Findings:

A radioimmunoassay system has been developed which is capable of measuring rhCG in biological materials and extracts of these materials. The RIA system utilizes a rat luteinizing hormone (rLH) tracer, an antiserum against human chorionic gonadotropin (hCG) and a house reference standard (HRP) obtained from the urine of a pregnant monkey. Urine extracts from pregnant animals dose out parallel to the standard, and sera from pregnant animals also can be assayed in this system. No response is obtained with urine or serum from normal non-pregnant animals or from castrate animals. The assay is 5 times more sensitive than present bioassays. In physiological studies, this system yields results comparable to those found by bioassay. However, the biological/immunological ratio approaches two.

Previous efforts to purify rhCG from urinary kaolin-acetone extracts utilizing polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing in polyacrylamide (IFPA) have been replaced by gel permeation chromatography and by ion-exchange chromatography on sephadex polymers. Estimation of effectiveness of purification procedures by rhCG RIA has made it possible to recognize those eluates of increased potency. Kaolin-acetone extracts were subjected to Sephadex G-100 gel filtration, cation-exchange Sephadex

chromatography and Sephadex G-200 gel filtration with a 30-40 fold increase in potency of a small fraction using the standard preparation (HRP) as reference material.

During the past three years, there has been disagreement as to whether the placenta of the rhesus monkey produces chorionic gonadotropin throughout pregnancy, and especially during the two to three weeks immediately preceding delivery. Another laboratory has published two studies showing increasing chorionic gonadotropin concentrations near the time of parturition. These studies have employed both bioassay and radioimmunoassay measurements. In contrast, we have been unable to detect any biological activity in placental extracts of term placentas either delivered naturally or obtained by C-section. Further, when kaolin-acetone concentrates of urine pools representing the entire week preceding natural delivery were tested in the mouse uterine weight assay (sensitive to 0.1 IU of hCG equivalents), no activity was detected. Currently, we are employing a radioimmunoassay for rhCG in these studies.

Common antigenicity of primate gonadotropins was tested in homologous hCG-α and hCG-β radioimmunoassay systems. The hCG-β system recognized common antigenic determinants in man, gorilla, and chimpanzee but not rhesus monkey. Except for the rhesus monkey, primate chorionic gonadotropins gave responses in the hCG-α system. However, evidence for non-identity was found.

A series of pregnant rhesus monkeys was ovariectomized at 22 to 24 days gestation. When the patterns of plasma estrogens (estradiol-17β and estrone) and progesterone were compared with intact controls, it was found that at about gestation day 35, estradiol-17β and estrone levels, which had been undetectable in ovariectomized monkeys and at basal levels in intact controls, rose to peak levels at about 80 days and maintained these levels until parturition in both groups. Throughout most of this period, the plasma concentration of estradiol-17β exceeded that of estrone by 4-5 fold. Likewise, progesterone levels resembled those seen in intact animals. Thus, production of these hormones from approximately 25 days of gestation to parturition must be exclusively from sources other than the ovary (placenta, adrenals). With expulsion of the placenta at parturition, estrogen and progesterone concentration declined to basal levels indicating that the placenta was the major source of these hormones.

From conception to day 25, peaks of estrogen were observed, one, just before ovulation, the other at about day 20 of pregnancy. On the basis of earlier findings, the latter surge of estrogen may derive from the corpus luteum in response to increasing levels of chorionic gonadotropin. The ratio of estradiol-17β to estrone is approximately one in this early period of gestation. Since we had previously shown that pregnancy cannot be maintained in the absence of ovaries prior to gestation day 22, the evidence points to an ovarian source of these steroids. Levels of rhesus chorionic gonadotropin were similar in ovariectomized and intact monkeys throughout the brief period of its production.

Light microscopic and fine structural observations were made on the corpus luteum of late pregnancy. Emphasis was placed on the granulosa lutein cells.

These cells are 25 to 30 μ in diameter and their plasma membranes are bordered by microvilli. The cytoplasm contains high concentrations of: 1) rod-shaped mitochondria with tubular cristae, 2) elaborate Golgi complexes and associated large tubular cisternae, 3) tubular as well as cisternal agranular endoplasmic reticulum, 4) well-developed parallel arrays of granular endoplasmic reticulum, 5) bundles of 50 Å filaments and 6) dispersed lipid droplets. Large intracellular lacunae are present in the cytoplasm of most cells. It was concluded that the morphological features of the granulosa-lutein cells of late pregnancy are consistent with steroid synthesis.

Proposed Course of the Project:

FSH is being purified from extracts of monkey urine. This will be used to develop specific antibodies. Heterologous systems for immunoassay have been developed and are being validated.

Purification of rhCG will be continued and the gestational events associated with CG secretion will be studied. The role of maternal FSH in follicular development of the fetus will be examined.

Serial No. HD-LB3
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Analytical Techniques and Instrumentation
a. Amino Acid Analysis
b. Automatic Monitoring of Chromatographic Experiments

Previous Serial Number: Same

Principal Investigator: E. Gross

Other Investigators: M. Morishita
J. Brown
J. L. Morell

Cooperating Units: Systems Maintenance Section DRS-BEI, NIH
Division of Computer Research and Technology, NIH

Man Years:

Total: 5
Professional: 3
Others: 2

Project Description:

Objectives:

1. Determination of the amino acid composition of peptides and proteins;
2. Improvement of existing amino acid analyzers and automation of techniques otherwise requiring many time-consuming operations;
3. Adaptation of conventional amino acid analyzers to the separation and quantitation of unusual amino acids of microbial origin;
4. Design, development, and operation of automated multiple sample applicators, the final instrument to offer adequate capacity for the continuous operation for prolonged periods of time without attendance.

Methods Employed:

Ionexchange chromatography on custom resins of spherical shape, colorimetry, and electronic data processing equipment.

Major Findings:

The second modified and improved sample applicator has been perfected to the point that it now operates on a 24-hour basis and performing 4-6 amino acid analyses depending upon the mode of operation. This facilitates greatly the handling of large numbers of amino acid analyses with amino acids of unusual structural features from membrane-active peptides, such as nisin, subtilin, cinnamycin, and duramycin. In the latter two peptides the presence of two new amino acids, β-hydroxyaspartic acid and lysinoalanine, necessitated adaptation of the one-column program. These amino acids are now well separated and quantitatively determined.

For the amino acid analysis of samples frequently available in only small quantities, one analyzer has been modified by electronic signal amplification for measurement of amino acids at the level of a fraction of a nanomole.

Significance to Biomedical Research and the Program of the Institute:

The structural elucidation of peptides and proteins requires large numbers of amino acid analyses. The extensive use of the amino acid analyzers with automatic sample application has considerably facilitated the structural elucidation of nisin, subtilin, cinnamycin, and duramycin, all peptides with unusual amino acids. Unique degradation products of these peptides are also quickly determined by related approaches. Nisin and fragments of nisin are capable of inducing fetal resorptions in rats. The precise knowledge of the mode of action of these peptides will only be possible after characterization of the active peptides with the help of numerous amino acid analyses.

Proposed Course of Project:

(1) The refinement of the design features of the applicators will be continued. Amino acid analyzers equipped with automated sample applicators will be linked to small independent computers capable of feedback and process control in order to demonstrate advanced techniques of laboratory automation and their application to protein chemistry. (2) The hydrolyses of peptides and proteins will be automated, and samples directly advanced to the automatic sample applicator for column loading. (3) Other laboratory processes will be further automated in collaboration with the Division of Computer and Research Technology. (4) The sequence analysis of peptides and proteins will be automated (a) by utilizing solid supports and (b) employing mass spectroscopic techniques. (5) Sensitivity in amino acid analysis will be further increased by (a) colorimeter (modification inclusive of constant temperature accomodation; (b) the use of ionexchange columns of lower diameter; and (c) modifications in reagents and buffers and their adequate accomodation.

Serial No. HD-LB14
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cell Organelles from Human Placenta: Formation, Isolation, Characterization, and Function Under Normal and Abnormal Conditions

Previous Serial Number: Same

Principal Investigator: E. Gross

Other Investigators: L. Corash
W. Gerber

Cooperating Units: I. Boime, Washington University, School of Medicine, St. Louis, Missouri

Man Years:

Total: 2
Professional: 2
Others: 0

Project Description:

Objectives:

1. Isolation and characterization of cell organelles, primarily of lysosomes;
2. Study of the membrane of lysosomes: chemical composition, reactivity, permeability, structural enzymes;
3. Constituents of lysosomes: isolation and characterization of enzymes, basic proteins, and other components;
4. Correlation of lysosomal enzymes and their function with the life cycle of the cell under normal and pathological conditions;
5. Analysis of lysosomal enzyme levels in the human placenta at various stages of gestation and the role they may play in human fetal development;
6. Preparation of a cell-free system of protein synthesis using lysosomes isolated from first trimester human placenta.

Methods Employed:

Ultracentrifugation, zonal centrifugation, carrier-free electrophoresis, techniques of enzyme analysis, fluorescence and electron microscopy, separation techniques for biopolymers.

Major Findings:

The syncytium is the major source of lysosomes in human term placenta. There are at least two different populations of lysosomes, one of which is associated with the endoplasmic reticulum. Acid phosphatase is not a suitable marker for human term placental lysosomes. The glucose-6-phosphate hydrolyzing activity of human term placenta is not identical with the conventionally defined glucose-6-phosphatase. Peroxisomes are not present in human term placenta. The syncytium of first trimester human placenta is also rich in lysosomes and contains two populations of the organelle. The latency of arylsulfatase of first trimester human placental lysosomes is different from that of the other acid hydrolases. First trimester human placenta is six to eight times richer in lysosomes than human term placenta. Vital staining of tissue from first trimester human placenta demonstrates the effect of nisin on lysosomes. Pretreatment with the peptide or its H_2N-terminal fragment abolishes the uptake of the vital stain.

Significance to Biomedical Research and the Program of the Institute:

Lysosomes are of central significance throughout the life cycle of the cell. The large number of hydrolases present in the organelle perform numerous important functions under normal and abnormal conditions. Profound physiological events, such as those of reproduction, may depend upon the availability of lysosomal enzymes. Extensive investigations of lysosomes in cells from different organs of the maternal as well as the fetal organism will contribute to the better understanding of the role of this organelle during development.

Proposed Course of Project:

(1) Improvement and application of techniques for the isolation of lysosomes from different types of developing tissue; (2) Isolation and characterization of lysosomal enzymes and other protein constituents from the organelle; (3) Elucidation of the structure and function of the lysosomal membrane; (4) Study of lysosomes under normal and abnormal conditions; (5) Investigation of the effect of low molecular weight peptides and proteins on the lysosomal membrane; (6) Correlation of the structure of membrane-active compounds with structural features of the lysosomal membrane; (7) Investigation of the enzyme content of human placental lysosomes at various stages of gestation.

Serial No. HD-LB15
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Fetal Resorption

Previous Serial Number: Same

Principal Investigator: E. Gross

Other Investigators: None

Cooperating Units: K. C. Snell, Laboratory of Pathology, National Cancer Institute

Man Years:

Total: 1/12
Professional: 1/12
Others: 0

Project Description:

Objectives:

1. Isolation, purification, and structural elucidation of substances capable of inducing fetal resorption;
2. Correlation of the structure and mode of action of these compounds;
3. Identification of the cellular constituents affected by and/or contributing to fetal resorption.

Methods Employed:

(1) Microbial culturing techniques to provide compounds with effects on the feto-placental unit, (2) chromatographic and counter-current distribution techniques in purification steps, (3) enzymatic and nonenzymatic methods in combination with amino acid analysis to determine the structure of peptides affecting fetal development, (4) animal experiments to test the activity of substances for which an effect on fetal development has been predicted, (5) characterization of tissue samples from the feto-placental unit, cell biological characterization to determine the mode of action of fetal resorption inducing agents after, for instance, radiolabeling by synthetic approaches.

Major Findings:

Nisin and H_2N-terminal fragments of nisin induce fetal resorption in rats when administered intravenously. Via the same route, these two compounds are also effective in preventing implantation by an as yet unknown mechanism. The COOH-terminal fragment of nisin is not effective. Its enzymatic degradation and inactivation is a likely event following intravenous injection. This is apparently also the case for nisin itself when it is administered orally. The gramicidins A, B, and C induce fetal resorption and prevent implantation when given orally.

Significance to Biomedical Research and the Program of the Institute:

The regulatory mechanisms operative at many stages of intrauterine development are not fully understood. Studies with substances of novel types of structures may serve to (1) identify physiological processes that contribute to the normal course of pregnancy, (2) detect undesirable metabolites which affect gestation adversely, and (3) offer chemotherapeutic approaches to pregnancy regulation.

Proposed Course of Project:

The substances indicated are being tested in a contract research laboratory. After the completion of these screening procedures if warranted, the following studies will be conducted: (1) Structural elucidation of peptides which affect fetal development; (2) correlation of structure and function during this physiological event; (3) synthesis of peptides in which the structural elements of nisin and the physiological properties of the gramicidins are combined; (4) investigation of dose levels, routes of administration, and timing of administration of substances that affect fetal development; (5) study of the effect of previously encountered resorptions on subsequent pregnancies; and (6) extension of these studies to primates.

Serial No. HD-LB5
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Synthesis of Biologically Active Peptides

Previous Serial Number: Same

Principal Investigator: E. Gross

Other Investigators: K. Noda

Cooperating Units: E. Blout, Harvard Medical School, Boston, Massachusetts

Man Years:

Total: 14/12
Professional: 14/12
Others: 0

Project Description:

Objectives:

1. Synthesis of peptides with biological activity, such as:
   a. effects on fetal development, and
   b. hormone releasing properties;
2. Testing and evaluating the biological properties of synthetic peptides.

Methods Employed:

(1) Conventional techniques of peptide synthesis, (2) solid phase methods of peptide synthesis, (3) chromatographic and countercurrent distribution techniques for separation and purification, (4) animal experiments and bioassays in the biological evaluation of the synthetic products, (5) radioimmunoassays in screening procedures; and (6) nuclear magnetic resonance and mass spectroscopic analysis of peptides.

Major Findings:

Synthesis via solid phase techniques of: Luteinizing hormone-follicle stimulating hormone releasing factor (LH/FSH-RF); H_2N-terminal tripeptide amide of LH/FSH-RF; the peptide has less than 0.1% of the activity of the natural releasing factor. A new solid phase technique, developed to

facilitate the synthesis of peptide amides, is based on the chemistry of α,β-unsaturated amino acids and utilized the experience gained during the structural elucidation of nisin and subtilin.

Significance to Biomedical Research the Program of the Institute:

The highly hydrophobic gramicidins A, B, and C constitute a unique group of peptides. Their alternating pattern of L and D-amino acids allows the formation of specific types of helices which, in turn, are capable of acting as ion-transporting transmembrane channels. The gramicidins A, B, and C affect fetal development in the rat. They also interfere with implantation. The hormone-releasing factors are of great interest as potential fertility regulating agents.

Proposed Course of Project:

(1) Synthesis of analogues of gramicidin A, B, and C; (2) synthesis of radio-labeled gramicidins; (3) synthesis of nisin, fragments of nisin, and analogs of these peptides with and without radioactive amino acids; (4) synthesis of hormone-releasing factors of peptide structure; (5) synthesis of subtilin, fragments of subtilin, and analogs of the parent molecule and partial structures; development of techniques for peptides synthesis.

Serial No. HD-LB19
1. Reproduction Research Branch
2. Section on Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Chemical Modification of Amino Acids. Selective Nonenzymatic Cleavage of Peptide Bonds. Structure-Function Relationship of Biologically Active Peptides and Enzymes

Previous Serial Number: Same

Principal Investigator: E. Gross

Other Investigators: J. L. Morell
M. Morishita
J. Brown
E. Nebelin
H. C. Chen

Cooperating Units: L. C. Craig, Rockefeller University, New York, New York; F. H. White, Jr., Laboratory of Biochemistry National Heart and Lung Institute; E. D. Becker, Laboratory of Physical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases; E. Scoffone, University of Padova, Padova, Italy

Man Years:

Total: 4
Professional: 4
Others: 0

Project Description:

Objectives:

1. Chemical modification of reactive groups in polyfunctional amino acids;
2. Selective cleavage of peptide bonds (nonenzymatic fragmentation) by application of chemical rea
3. Production, isolation, and purification of biologically active peptides and enzymes;
4. Fragmentation of biologically active peptides and enzymes with the objective of studying residual activity;
5. Synthesis of enzyme inhibitors and study of inhibitory effects, identification of the site of inhibition and of loci responsible for enzyme activity;

6. Structural elucidation of biologically active peptides and correlation of structure and function.

Methods Employed;

(1) Enzymes; (2) chemical reagents for nonenzymatic fragmentation; (3) high energy radiation techniques; (4) photochemical techniques; (5) advanced separation and analytical techniques: chromatography, electrophoresis, countercurrent and counter double current distribution, automatic sample application in the amino acid analysis, electronic equipment in data acquisition and processing; (6) nuclear magnetic resonance; (7) mass spectroscopy; and (8) culturing techniques and bioassays.

Major Findings:

(1) Nisin: The α-aminobutyric acid moieties of the β-methyllanthionine residues and the alanine moiety of lanthionine first in the linear sequence are of the D-configuration. This indicates a stereospecific step in the biosynthesis of the peptide. (2) Subtilin: The structure of subtilin has been established as that of a heterodetic pentacyclic peptide of 32 amino acids. Nisin and subtilin, thus, have in common the higher structural principle of five heterodetic peptide cycles all of which are identical in their respective size. (3) Cinnamycin and duramycin are closely related peptides with lanthionine and β-methyllanthionine. Dehydroalanine is present in these molecules in the masked state of lysinoalanine. The two peptides resist enzymatic degradation and nonenzymatic fragmentation methods are necessary for their structural elucidation. (4) High energy radiation of the COOH-terminal fragment of nisin in the presence of tritium incorporates the label with preference in histidine and serine, a fact indicating a distinct role of these two amino acid residues. (5) The synthesis of human chorionic gonadotrophin in trophoblast culture is affected by nisin. (6) Nisin and nisin fragments undergo photochemical changes which are likely to originate with the α,β-unsaturated amino acids. (7) Sizeable quantities of nisin and nisin fragments have been purified for studies of their effects on lysosomes and on fetal development. (8) Pepsinogen has been fragmented by consecutive application of the cyanogen bromide reaction at methionine and S-methylcysteine. The resulting fragments have been separated and purified to finally cleave the products of the pseudo-N→O-acyl shift. (9) The bromate/bromide oxidation reaction is applicable to the fragmentation of peptides by cleavage of the tryptophyl peptide bond. (10) Esterification of terminal COOH-groups followed by reduction to the alcohol and N→O-acyl shift constitute a route to the stepwise removal of COOH-terminal amino acid residues.

Significance to Biomedical Research and the Program of the Institute:

The detailed knowledge of the structure of nisin, and of other peptides with α,β-unsaturated amino acids is of great importance in view of the biological properties of these biopolymers. The implied working hypothesis of the interaction of α,β-unsaturated amino acids with vital sulfhydryl groups may well be of broad biological significance. This reaction is reversible and linked to the formation of ketoacids and amides, frequently encountered

constituents of many biological systems. These structural considerations form the basis for the study of the effect of nisin and related peptides on fetal development.

Proposed Course of Project:

(1) Continued exploration and application of nonenzymatic methods to the structural elucidation of peptides and proteins: (a) application of selective oxidants to the cleavage of tryptophyl peptide bonds; (b) study of the selective suppression of the cleavage of methionyl peptide bonds upon treatment with cyanogen bromide; (c) fragmentation of reduced and S-alkylated peptides and proteins; fragmentation of peptides and proteins subsequent to mercaptan addition to α,β-unsaturated amino acids; direct fragmentation at α,β-unsaturated amino acids, at times preceded by β-elimination; (d) the fragmentation at aspartic acid residues after esterification and reduction to be developed to a generally applicable method of nonenzymatic fragmentation. (2) Nisin: Continued structural elucidation of nisin-like peptides from Streptococcus lactis. (3) Subtilin: Elucidation of the configuration of the β-carbon atoms of β-methyllanthionine and determination of the isomerism in dehydrobutyrine study of phylogenetic aspects of the structure of peptides containing lanthionines and α,β-unsaturated amino acids. (4) Cinnamycin and Duramycin: Structural elucidation and structural correlation. (5) Preparation of peptides with activities on fetal growth.

Honors and Awards:

Mortimer B. Lipsett

Invited Lectures

International Cancer Meeting, Yugoslavia, August 1972
University of Paris, September 1972
University of Copenhagen, September 1972
Endocrine Society, Post-graduate Assembly, Birmingham, October 1972
Annual Meeting, Brazilian Endocrine Society, Brazil, November 1972
American College of Physicians, Brown University, Providence, November 1972
College of Physicians and Surgeons, Columbia University, New York, February 1973
American College of Physicians, New York, March 1973
New York Academy of Science, New York, March 1973
Massachusetts Institute of Technology, Cambridge, April 1973
Serono Foundation, Course in Endocrinology, Florence, April 1973
American College of Physicians, Ann Arbor, May 1973

Technical Review Committee, Male Reproduction, World Health Organization, 1972-
Chairman, Post-graduate Teachers Assembly, Endocrine Society, 1973-
Committee on Endocrinology and Metabolism, Food and Drug Administration 1973-
Elected Corresponding Member German Endocrine Society, 1973-

G. T. Ross

Invited Lectures

John E. Fogarty International Center and League for the Fight Against Cancer of the Croatian Republic of Yugoslavia, Yugoslavia, August 1972
College of Physicians and Surgeons, Columbia University, New York, September 1972
Airlie Foundation, Airlie, Virginia, October 1972
Late Effects Workshop Meeting, Children's Cancer Research Foundation, Boston, October 1972
Harold C. Mack Symposium, Wayne State University and Harper Hospital, Detroit, November 1972
American College of Physicians Post Graduate Course, Johns Hopkins Medical Institutions, Baltimore, November 1972

Awards Committee, Endocrine Society, 1972
Endocrine Study Section, 1972
Breast Cancer Task Force, Treatment Committee, 1972
Scientific Advisor to the Medical Research Institute of Worcester, Inc., Worcester, 1972-
Committee on Standards of the International Society of Endocrinology, 1973-

Wm. W. Tullner

Consultant in Endocrinology and Metabolism, The George Washington University Graduate School, Washington, D. C.

Research Associate, Department of Zoology, Graduate School, Howard University, Washington, D.C.

Erhard Gross

Invited Lectures

Third American Peptide Symposium, Boston, June 1972
Ninth International Congress of Biochemistry, Stockholm, June 1973

K. J. Catt

Invited Lectures

Vanderbilt University, Nashville, July 1972
Worcester Foundation for Experimental Biology and Medicine, Shrewsbury, February 1973
George Washington University, Washington, D. C., February 1973
University of Florida, Gainesville, March 1973
University of California, Los Angeles, March 1973
Department of Nuclear Medicine, Johns Hopkins University, Baltimore, March 1973
University of Milan, May 1973

Member, American Society for Clinical Investigation (Young Turks), May 1973

M. L. Dufau Catt

Invited Lectures

University of Milan, May 1973

A. Chrambach

Invited Lectures

International Conference on Isoelectric Focusing and Isotachophoresis, New York, May 1972
Gordon Research Conference, New Hampton, June 1972
Conference on Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel, Tubingen, Germany, October 1972

G. D. Hodgen

Invited Lectures

Satellite Symposium of IV International Congress of Endocrinology, Washington, D.C., June 1972

P. O. Kohler

Fellow, American College of Physicians, October 1972
Member, American Society for Clinical Investigation (Young Turks), May 1973

H. B. Pollard

Invited Lectures

University of Chicago, November 1972
Membrane Conference, Squaw Valley, March 1973
University of California, Berkeley, March 1973
University of California, San Francisco, March 1973
California Institute of Technology, Pasadena, March 1973
Johns Hopkins University, Baltimore, April 1973
Oxford University, Laboratory of Molecular Biophysics, Oxford, May 1973
International Catecholamine Congress, Strasbourg, May 1973
Washington Crystal Colloquim, June 1973

D. Rodbard

Invited Lectures

Vanderbilt University, Nashville, July 1972
University of Colorado and Veterans Administration Hospital, Denver, September 1972
Max-Planck Institute, Tubingen, October 1972
Stanford University Medical School, Palo Alto, December 1972
University of California Medical Center, San Francisco, December 1972
University of Washington Medical School and American Association of Clinical Chemists, Seattle, December 1972
University of California Medical School, Los Angeles, December 1972
Harbor General Hospital, Torrance, December 1972
Wadsworth Veterans Administration Hospital, Los Angeles, December 1972
Kroc Foundation for the Advancement of Sciences, Santa Barbara, April 1973

R. J. Sherins

Invited Lectures

Columbia Presbyterian Hospital, New York, September 1972
Hershey Medical Center, Hershey, October 1972
Holy Cross Hospital, Silver Spring, January 1973
George Washington University Medical School, Washington, D.C., February 1973
Washington Pediatric Society, March 1973

Fellow, American College of Physicians, 1973

J. L. Vaitukaitis

Invited Lectures

University of Maryland School of Dentistry, Baltimore, February 1973
American Fertility Society Postgraduate Course, San Francisco, April 1973
George Washington School of Medicine, Washington, D.C., January & February 1973
University of California, San Diego, November 1972
Boston University School of Medicine, Boston, April 1973

PUBLICATIONS

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Ben-David, M., Rodbard, D., Bates, R.W., Bridson, W.E., and Chrambach, A.: Human prolactin in plasma, amniotic fluid and pituitary identity and characterization by criteria of electrophoresis and isoelectric focusing in polyacrylamide gel. J. Clin. Endocrinol. Metab. 36: 135-148, 1973.

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Berman, M.L., Hanson, K., and Hellman, I.L.: Effect of breast-feeding on postpartum menstruation, ovulation, and pregnancy in Alaskan Eskimos. Am. J. Obstet. Gynecol. 114: 524-534, 1972.

Braunstein, G.D., Bridson, W.E., Glass, A., Hull, E.W., and McIntire, K.R.: In vivo and in vitro production of hCG and alpha-fetoprotein by a virilizing hepatoblastoma. J. Clin. Endocrinol. Metab. 35: 857-862, 1972.

Braunstein, G.D., Grodin, J.M., Vaitukaitis, J., and Ross, G.T.: Secretory rates of hCG by normal trophoblast. Am. J. Obstet. Gynecol. 115: 447-450, 1973.

Braunstein, G.D., and Kohler, P.O.: Pituitary function in Hand-Schuller-Christian Disease. New Engl. J. Med. 286: 1225-1229, 1972.

Braunstein, G.D., Vaitukaitis, J.L., Carbone, P.P., and Ross, G.T.: Ectopic production of hCG by neoplasma. Ann. Intern. Med. 78: 39-45, 1973.

Braunstein, G.D., Vaitukaitis, J.L., and Ross, G.T.: The in vivo behavior of hCG after dissociation into subunits. Endocrinology 91: 1030-1036, 1972.

Cain, M.D., Coghlan, J.P., and Catt, K.J.: Measurement of angiotensin II in blood by radioimmunoassay. Clin. Chim. Acta 39: 21-34, 1972.

Cantz, M., Chrambach, A., Bach, G., and Neufeld, E.F.: The Hunter corrective factor. J. Biol. Chem. 247: 5456-5462, 1972.

Cargille, C.M., Vaitukaitis, J.L., Bermudez, J.A., and Ross, G.T.: A differential effect of ethinyl estradiol upon plasma FSH and LH relating to time of administration in the menstrual cycle. J. Clin. Endocrinol. Metab. 36: 87-94, 1973.

Catt, K.J., Dufau, M.L., and Tsuruhara, T.: Absence of intrinsic biological activity in LH and hCG subunits. J. Clin. Endocrinol. Metab. 36: 73-80, 1973.

Catt, K.J., Tsuruhara, T., and Dufau, M.L.: Gonadotrophin binding sites of the rat testis. Biochim. Biophys. Acta 279: 194-201, 1972.

Catt, K.J., Watanabe, K., and Dufau, M.L.: Cyclic AMP released by rat testis during gonadotrophin stimulation in vitro. Nature 239: 280-281, 1972.

Chrambach, A., Hearing, E., Lunney, J., and Rodbard, D.: Experimental validation of the predicted properties of a multiphasic buffer system applied to polyacrylamide gel electrophoresis. Separation Sci. 7: 725-745, 1972.

Chrambach, A., Kapadia, G., and Cantz, M.: Isotachophoresis on polyacrylamide gel. Separation Sci. 7: 785-816, 1972.

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REVIEWS AND CHAPTERS

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NICHD Annual Report
Behavioral Biology Branch
July 1, 1972 through June 30, 1973

Section on Brain and Behavior

The professionals in this section are Drs. David Symmes and John Newman. Mrs. Garland Riggs has contributed through her duties on a professional services contract.

1. Drs. Newman and Symmes have shown that the responses of single neurons in the primate auditory cortex to complex biologically significant acoustic stimuli are little affected by wide fluctuation in arousal level of the animal. The complex synaptic connections responsible for the relatively specific responses to such stimuli as species-specific vocalizations, therefore, represent securely organized sensory channels, rather than some diffuse system related to attention or arousal.

2. Drs. Symmes and Newman completed two studies on the discrimination of vocalizations by squirrel monkeys in a behavioral situation. One group of monkeys performed quite well in differentially responding to two, quite similar variants of natural isolation peeps. The discriminations were based on relatively subtle aspects of these natural calls, since simple variation of loudness or duration could be shown not to be involved in the discrimination. Another group of monkeys, while discriminating between natural and computer generated isolation peeps did not discriminate between dissimilar pairs of computer generated isolation peeps. These results strongly indicate that natural calls contain distinctive but subtle features to which the natural discriminative systems are peculiarly acutely attuned.

3. Extensive sound spectrographic analysis of the vocalization of one pair of squirrel monkeys indicate that each animal has a distinctive fine structure in its vocal productions. Call duration, number of syllables and complexity of syllable structure all seem to differentiate and made unique an individual monkey's vocal repertoire.

4. Dr. Symmes has completed histological verification of his studies of brain electrical activity in isolation reared monkeys. The histological evidence generally supports the conclusion that abnormal electrical activity was recorded from the vicinity of the frimbria and dentate gyrus of the hippocampus. Some placements were slightly dorsal to the hippocampus, but close enough to be consistent with evidence that stimulation of these sites induced hippocampal after-discharges. The higher placements rule out possible damage to the hippocampus itself and therefore argue against an irritative lesion as a basis for the observed electrical abnormalities. Computer analysis confirmed in an objective quantitative way the previously reported nystagmus related electrical abnormalities in the isolates. The limbic sites on the side opposite the calorically stimulated vestibular apparatus were more involved than ipsilateral sites.

Section on Neurobiology

The professionals in this section include Drs. Phillip Nelson, Harold Gainer, Gerald Fischbach, Bruce Schrier, Earl Giller, Ronald Fisk, Anthony Breuer, John Whysner, Maryanna Henkart, Jeffery Barker, Stephen Cohen, Clifford Christian and Asher Shainberg.

1. Drs. Harold Gainer and Jeffery Barker have used the biochemical analytical methods developed by Dr. Gainer in an extensive study of individual neuronal synthetic patterns in the molluscan nervous system. Individual neurons have distinctive patterns of protein synthesis and one class of neurons exhibited sufficiently high turnover rates to warrant a study of the effects of electrical and synaptic activity on their synthesis patterns. Synaptic inhibition reduced the rate of incorporation of radioactive leucine into two low molecular weight neurosecretory proteins by 50%. Dopamine, the presumed transmitter mediating the synaptic inhibition produced similar reduction in synthesis of the neurosecretory protein. The synthesis rates of other proteins in the cell studies were not significantly influenced by either synaptic inhibition or dopamine. Both these treatments increase membrane potential and increase surface membrane permeability to potassium. High potassium solutions increase potassium permeability but lower membrane potential and this treatment produces a selective doubling of the synthesis rate of the low molecular weight proteins. This argues against the increase in potassium permeability being the causative control event and the possibility that an increased membrane potential might be directly involved in control of protein synthesis is currently under investigation. Other possible factors under consideration are changes in intracellular calcium or sodium ions.

Studies on protein synthesis in squid axons indicate that Schwann cells are the site for synthesis of a wide range of proteins which are subsequently found in the axon. The regulatory mechanisms governing this glial-neuronal interaction are now under study.

Divalent cations have been shown to play a major regulatory role in both the bursting pacemaker activity of molluscan neurosecretory cells and the seasonal modulation of this pacemaking activity. Sterols also are involved in regulation of this behavior; 25-hydroxycholecalciferol (a vitamin D metabolite) reversibly inhibits the pacemaker activity of these cells. Naturally occurring sterols from the molluscan nervous system in normal and aestivated animals are being investigated to see whether these materials may play some role in regulating neural behavior in these animals. Studies on the mechanisms of the bursting pacemaker potentials in the neurosecretory cells (in collaboration with Dr. T. Smith, Laboratory of Neurophysiology, NINDS) indicate that this activity is primarily due to two membrane properties 1) a voltage dependent inward divalent and monovalent cation current closely coupled to 2) a voltage-dependent outward potassium current.

These combined electrophysiologic and biochemical approaches have defined an important class of membrane properties and show a close coupling between membrane events and synthesis of specific proteins within the cell.

2. These experiments involve Drs. Nelson, Peacock, Giller, Fisk and Shainberg. Mouse cerebellar neurons have been shown to survive dissociation and develop complex patterns of spontaneous and synaptically mediated electrical activity. Clearly distinguishable, morphologic types and a range of electrophysiologic characteristics were found in the surviving neuronal networks so that some substantial representation of the relatively simple normal cerebellar circuitry is presumably present in the culture systems. Mouse cerebellum is a favorable preparation for in vitro studies from the standpoint of the variety of genetic mutants with relatively well established neuropathologic abnormalities.

Interactions between nerve and muscle have been studied in cultures derived from dissociation of tissues of dystrophic chick embryos. Less than 25% of muscle cells in normal combined spinal cord-muscle culture became innervated but well over 50% of dystrophic muscle gave evidence of innervation when combined with either normal or dystrophic spinal cord cells. The possibility that this represents some defect in the "trophic" mechanism whereby multiple innervation is prevented needs to be investigated.

Calcium ions play an important role in active electrical membrane responses in both neuroblastoma cells and fibroblasts (L-cells). Action currents in the neural cells are partly carried by calcium ions and intracellularly injected calcium elicits the L-cell response with a very short latency. The various stimuli which can elicit this fibroblast response therefore probably work through the intracellular release of calcium. Since intracellular calcium release has been shown to be an important factor involved in the regulation of cell division and a variety of metabolic processes, these preparations are favorable for study of the coupling between surface membrane activity and intracellular metabolism.

Four to five fold enrichment for cell surface membranes has been obtained by subcellular fractionation. The neuroblastoma and one L-cell clone have been studied, with disc electrophoretic separation of total cellular and surface membrane proteins. While the resolution of the system appears to be good, and clear differences in the protein composition of neuronal and nonneuronal cell membranes have been found, substantially more data are required in different clones and with different growth conditions in order to begin to make the correlations between membrane structure and function which is the purpose of this work.

3. Drs. Fischbach and Cohen have studied factors important in the regulation and disposition of acetylcholine receptors on the surface of chick muscle fibers. Innervation of the muscle fibers does not change the general level of acetylcholine sensitivity, but muscle activity does have a pronounced effect on chemosensitivity. A high degree of ACh sensitivity all along the fiber surface characterizes muscle fibers in which all electrical and mechanical activity has been blocked by tetrodotoxin or lidocaine. Untreated spontaneously active fibers have somewhat lower ACh sensitivity and electrically stimulated fibers are less than one-tenth as sensitive as the treated inactive fibers. These changes in sensitivity are nearly complete within 24 hours. Morphologic study (by electron microscopy and by phase contrast or Nomarski cinematography) show a number of features that might account for the

variation in concentration of receptor molecules in the muscle surface. Coarse membrane folds, microvilli and an extensive tubulo-vesicular network that communicates with the extracellular space are likely candidates. Time-lapse cine studies have shown that muscle nuclei move up to 300 μ within muscle fibers at rates as fast as 40 μ/hr. This may explain some of the exceptions to the general rule that high ACh sensitivity is correlated with underlying muscle nuclei. Drs. Fischbach and Whysner have shown that ACh receptors of cultured muscle cells bind α-bungarotoxin in the same manner as receptors in other cells. Toxin binding sites increase as myoblasts fuse with one another and reach a plateau after 4-6 days. The irreversible toxin-receptor interaction is not altered when the muscle membrane is depolarized by high potassium solutions.

4. Dr. Schrier in collaboration with Dr. Alfred Gilman has continued a study of the accumulation of cyclic adenosine monophosphate stimulated by the addition of adenosine to cultures of rat brain cells or to neuroblastoma-L cell hybrids. This adenosine-stimulated cAMP accumulation, peculiar to brain of all body tissues which have been studied, is paraxodically inhibited by theophylline in both brain slices and brain cell cultures, but not in the hybrid cell lines. In the brain cell cultures, changes in plating density which increase choline acetyltransferase activity also increase the magnitude of the adenosine response while decreasing the rise in cAMP produced by catecholamines. Since glial cells exhibit the catecholamine response, it would appear that the adenosine response is likely to be a neuronal marker. The rat brain cell cultures have been shown to contain a large population of cells which generate typical neuronal action potentials and are synaptically coupled as well. Some cells in the cultures were found to contain the glial fibrillary acidic protein characteristic of certain brain astrocytes in vivo. These preparations, therefore, exhibit a full spectrum of neurobiologic phenomena.

Methods have been developed for a study of the synthesis, uptake, storage and excretion of the putative neurotransmitters γ-aminobutyric acid (GABA), taurine, β-alanine and of isethionic acid. Three glioma cell lines were studied and compared to a brain fibroblast cell line. GABA was synthesized by the glial cells from glutamic acid in the medium and GABA uptake occurred via a saturable component with an affinity constant of 10^{-5}M and a non-saturable component that was responsible for about 90% of total uptake at external concentration of 160 μM. The fibroblasts exhibited only the non-saturable component. All four cell lines showed 2-component uptake for taurine with saturable component affinity constants of 10^{-5}M and V_{max} values nearly 20-fold higher than those for GABA. The non-saturable component was relatively less significant for taurine than for GABA uptake reaching 30-45% for external taurine concentration of 200 μM. In experiments evaluating excretion rates it was found that cell/medium concentration ratios of 1000-fold would be maintained with respect to taurine. The data suggested that glial cells may modulate neuronal activity by regulating levels of neuroactive substances in the extracellular fluid.

5. Dr. Henkart has set up the electron microscopic facility in the Behavioral Biology Branch and has extended her earlier studies utilizing the EM to demonstrate cellular sites of calcium binding or accumulation. These studies

are designed to explore the possibility that certain physiologic, metabolic function in neurons and other cells are regulated in coordination with membrane activity via calcium entry or release of calcium from intracellular stores. She has found that the fine structure of the endoplasmic reticulum of the squid giant axon changes when the divalent cation composition of the external medium is altered and when membrane potential is changed. The relationships between subsurface cisternae of the ER and the surface membrane is a function of the concentration and type of divalent cation in the external medium. Similar effects have been seen in the ER of Aplysia neurons. The observations so far suggest an analogy between the ER of neuron and the sarcoplasmic reticulum of muscle, which is known to be a calcium-sequestering and releasing system. This system plays an important metabolic regulatory role in muscle, and if the analogy to the neuronal ER holds, it will be an important area for study in neuronal and other cell types.

Serial No. HD-BB2
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies of CNS functioning in isolation reared monkeys.

Previous Serial Number: Same

Principal Investigator: David Symmes

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.25
Professional:	0.25
Others:	0.0

Project Description:

Objectives: To describe alterations in CNS function, if any, which are associated with the isolation-reared syndrome in monkeys.

Major Findings: Data collection was completed at the end of FY 1972. The work done on this project during FY 1973 was limited to preparation and study of histological material from the brains of the implanted monkeys, and to computer analysis of the nystagmus related abnormality observed in the isolation reared group.

The histological evidence generally supports the interpretation that the spontaneous abnormalities were recorded from the vicinity of the fimbria and dentate gyrus of the hippocampus. Some placements were slightly dorsal to the hippocampus but close enough to indicate that stimulation of these cells induced after discharges of the hippocampus. The higher placements rule out possible damage to the hippocampus itself and therefore eliminate an irritative lesion explanation of the EEG abnormalities observed.

Computer analysis of the nystagmus related abnormality provided quantitative evidence in support of the previously reported difference between isolate and control monkeys. An additional unsuspected finding was an asymmetry of "nystagmus following" - the limbic sites on the side opposite to the irrigated ear were somewhat more involved than those on the ipsilateral side. It is possible that these data point to an anatomical pathway from vestibular to limbic structures which is abnormal.

Significance to Biomedical Research and the Program of the Institute: The severity and persistence of the behavioral abnormalities associated with isolation rearing are well documented, yet little or no data exists on the neurochemical or neurophysiological mechanisms which may be causally related. Some insight into these mechanisms could have wide applicability in the diagnosis and treatment of certain childhood disorders, particularly autism.

Proposed Course: The project has been inactive during most of FY 1973 in part due to the long lead time involved in securing additional monkeys and in part due to controversy related to interpretation of the findings, most of which were reported in last year's annual project report.

Honors and Awards: None

Publications: None

Serial No. HD-BB3 (1)
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Morphologic and biochemical studies of nervous system cells in culture.

Previous Serial Number: Same

Principal Investigator: Bruce K. Schrier, M.D., Ph.D.

Other Investigators: Phillip G. Nelson, M.D., Ph.D.
Earl L. Giller, M.D., Ph.D.

Cooperating Units: Alfred G. Gilman, M.D., Ph.D., Dept. of Pharmacology University of Virginia, School of Medicine, Charlottesville, Virginia
Samuel H. Wilson, M.D., Laboratory of Biochemistry, National Cancer Institute
A. Bignami, M.D., Veterans Administration Hospital, Pathology Research, Palo Alto, California

Man Years:

Total:	1.5
Professional:	1.0
Others:	0.5

Project Description:

Objectives: 1) Elucidate the mechanism of the adenosine stimulated accumulation of cyclic AMP in, and peculiar to, brain. 2) Further study of the development of nervous system biochemical and electrophysiological characteristics in the brain cell culture system. 3) Evaluate the importance of glial contributions to nervous system function. 4) Continue a search for biochemical markers of cell types in the nervous system.

Methods Employed: Methods for growing brain cells in culture and assaying for nervous system marker enzymes have been described previously. Assays for intracellular cyclic AMP were performed by the method of Gilman. Cells containing glial fibrillary acidic protein were identified by immunofluorescence by the method of Bignami, et al. Methods for detecting taurine and β-alanine synthesis by cells and for collecting and counting tritiated macromolecules with high efficiency were developed.

Major Findings: 1) Investigations of hormone-stimulated accumulations of cyclic AMP in brain have continued with a study of the response due to

adenosine in fetal rat brain cell cultures. The adenosine response, peculiar to brain, is characterized by an unexplained inhibition by theophylline and by adenosine potentiation of the response to norepinephrine. The characteristics of the adenosine response in brain slices were reproduced in the brain cell cultures, indicating that the cultures are a suitable simplified system for studying brain cyclic AMP metabolism. Changes in plating density which resulted in increased magnitude of the adenosine response also increased the activity of choline acetyltransferase, a neuronal marker enzyme, and decreased the magnitude of the catecholamine response. Since the latter has been shown to be, at least in part, due to glia, the data suggest that the adenosine response is a neuronal marker. In a survey of adenosine analogs, N^6-isopentenyladenosine and 2-chloroadenosine were found to produce a response similar to that stimulated by adenosine. The halogenated analog was more potent, produced a greater maximal effect, and was apparently operating by the same mechanism as adenosine. We are presently studying the metabolic fate of 2-chloroadenosine in brain cell cultures in order to determine whether the adenosine response is receptor-mediated and/or functions through provision of substrate for formation of cyclic AMP via substrate-limited or substrate-regulated pathways.

2) In related studies, a theophylline-stimulated adenosine response was observed in some established cultured cell lines (mouse neuroblastoma X L cell hybrids produced by Dr. J. Minna). These cells were similarly more responsive to 2-chloroadenosine than to adenosine and both responses were potentiated by theophylline. The investigation of the adenosine response in these cells is continuing in conjunction with the brain cell culture studies.

3) In order to determine the best method for assay of macromolecular synthesis by cultured cells, a study was made with Dr. Samuel H. Wilson of the optimal conditions for filtration collection and counting of acid-precipitated tritiated macromolecules. The following results were obtained using whole, high molecular weight (25×10^6 daltons) or sheared (mean, 185,000 daltons) bacteriophage T_7 [^{3}H]DNA. (a) Counting efficiences were much higher and more reliable when the DNA and co-precipitant protein were dissolved in the scintillation solution prior to counting. With two solubilization-counting techniques counting efficiencies were independent of the amount of co-precipitant protein. (b) Counting efficiencies depended markedly on protein content when solubilization was not effected. (c) DNA collected on, and solubilized from, glass fiber filters counted at much higher efficiencies than the highest efficiencies obtained with nitrocellulose filters. (d) With high molecular weight or sheared DNA, collection of DNA on nitrocellulose filters was complete without added protein; with glass fiber filters complete collection of all of the sheared DNA was not attained, regardless of the amount of co-precipitant protein. (e) The type of filter did not appear to affect the magnitude of the reaction "blank" (tritiated thymidine triphosphate retained by the filter). (f) Addition of competing small molecules to the precipitant and additional washes were found to lower this "blank", although the additional washes removed additional sheared DNA from glass fiber filters. It was concluded that macromolecules of small or unknown molecular weight should be collected on nitrocellulose filters and counted only after solubilization.

4) In order to evaluate the possibility that glioma cells might be capable of synthesis of the putative neurotransmitters γ-aminobutyrate (GABA), taurine, isethionic acid and β-alanine, methods were developed for assay for synthesis of these compounds by cells in culture. High voltage electrophoretic systems were devised which gave adequate separations of all the compounds from their precursors and from each other, except taurine from cysteine and cystine. Treatment of samples with bromine water was found to convert cystine and cysteine to cysteic acid, thus allowing assay of taurine as well.

5) In a continuation of the studies with glial tumor cell metabolism of certain putative neurotransmitters, the following additional observations were made. (a) C-6 cells in culture synthesized GABA and glutamine from glutamate in the medium and glutamine, but not GABA or β-alanine, from aspartate in the medium. (b) GABA uptake, identically for C-6, C-2_1 and NT-1, consisted of a saturable component with an affinity constant of about 10^{-5}M, and a non-saturable component which was responsible for 90% of total uptake at external concentrations of 160 μM. (c) RBF cells did not apparently have the saturable component of GABA uptake. (d) All four cell lines showed two-component uptakes for taurine with saturable component affinities of about 10^{-5} and V_{max} values nearly 20-fold higher than those for GABA. The non-saturable component was relatively less significant for taurine than for GABA uptakes, reaching 30% of total uptake for C-2_1, NT-1 and RBF cells and 45% for C-6 cells at external concentrations of 200 μM. (e) In experiments to evaluate excretion rates it was found that C-6 and C-2_1 cells could maintain cell/medium concentration ratios for taurine in excess of 1000-fold. These data, with those previously obtained, are consistent with the possibility that glial cells in the nervous system may modulate neuronal activity by control of the extracellular levels of some neuroactive substances.

6) Fetal rat brain cell cultures, treated with media containing inactivated horse serum and FUdR were found to contain cells which, by electrophysiologic criteria, resembled neurons. Evidence for the presence of functional synapses was also obtained. The cultures also contained cells which stained for the "glial fibrillary acidic protein" characteristic of certain brain astrocytes in vivo. Thus, each brain characteristic for which we have assayed has been found in the brain cell cultures.

Significance to Biomedical Research and the Program of the Institute: We are beginning to understand the development of the nervous system and some of its functions via studies with culture systems. These studies are significant to biomedical research in that they provide simplified systems with which such developmental events, in healthy and diseased cells, may be examined. Interactions between various cell types in the central and peripheral nervous systems are of basic relevance to neurobiology. In addition, an understanding of the genetic and biochemical control mechanisms necessary to regulate and/or modify these interactions will contribute significantly to the diagnosis and treatment of nervous system diseases.

Proposed Course: The following studies are actively continuing. 1) Mechanism of the adenosine-mediated cyclic AMP response in brain cell cultures, 2) Biochemical and electrophysiologic events and controls associated with the development of neuromuscular junctions in cultured cells. 3) A continued

search for new markers of nervous system function and new cell culture systems in which marker control systems may be investigated. 4) Further evaluation of biochemical and electrophysiologic developmental phenomena in cultured brain cells.

Honors and Awards: None

Publications:

1. Schrier, B. K.: Surface culture of fetal mammalian brain cells: effect of subculture on morphology and choline acetyltransferase activity. J. Neurobiol. 4: 117-124, 1973.

2. Shapiro, D. L. and Schrier, B. K.: Cell cultures of fetal rat brain: growth and marker enzyme development. Exptl. Cell Res. 77: 239-247, 1973.

3. Shapiro, D. L.: Morphological and biochemical alterations in fetal rat brain cells cultured in the presence of monobutyryl cyclic AMP. Nature 241: 203-204, 1973.

Serial No. HD-BB4
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Information processing in the central auditory system of mammals.

Previous Serial Number: Same

Principal Investigator: John D. Newman, Ph.D.

Other Investigators: David Symmes, Ph.D.

Cooperating Units: Mrs. Garland Riggs

Man Years:

Total:	2.25
Professional:	1.75
Others:	0.5

Project Description:

Objectives: This project is concerned with the processing of complex sounds by the primate cerebral cortex and related brain structures. Special attention is being paid to sounds used by primates in intra-specific communication, to determine which cues are used by the brain and the animal to distinguish between the various elements of the communication repertoire.

Methods Employed and Major Findings: 1) Single unit recording: Action potentials are recorded from single cells in the auditory cortex of unanaesthetized squirrel monkeys. Neuronal discharges are analyzed for excitatory or inhibitory effects of vocal stimuli. This year we have investigated the role of arousal level in influencing response patterns to species-specific vocalizations. In this study we monitored surface EEG and altered arousal level via electrical stimulation of electrodes implanted in the midbrain reticular formation. The principal finding was that despite widespread fluctuations in arousal (as judged from computed analysis of EEG) the response properties of single auditory neurons remained relatively constant. Neither spontaneous discharge rate nor responsiveness to vocal stimuli correlated with arousal level in the great majority of units studied. A small number of units, however, were found to be influenced by reticular stimulation at current levels sufficient to produce behavioral and EEG arousal in drowsy monkeys. These included units which became responsive to previously ineffective vocal stimuli, and others which were restricted or weakened in their responses. These findings suggest that the complex feature detection properties of auditory cortex neurons are not characterized by

great plasticity during shifts in arousal level.

2) Behavioral training: Two studies were completed relating to the capacity of squirrel monkeys to discriminate between naturally occurring variations in one call type, the isolation peep. This call type was chosen because of its demonstrated semantic significance (restoration of troop cohesion during periods of obstructed visual contact), because of its relatively simple acoustic structure, and because of our finding that auditory neurons can discriminate between isolation peep variants. The first study attempted to test the hypothesis that natural variations of this call type are behaviorally meaningful to the monkey. Monkeys were trained via shock avoidance techniques to jump from one perch to another when one isolation peep variant was presented, and to refrain from jumping when a second variant was presented (i.e., a go-no go shuttle task). Three monkeys mastered this task readily. The fourth monkey did not attain the 90 percent correct performance level of the other three, but did demonstrate better than chance discrimination. The hypothesis in question was tested and confirmed by continuing the training with new isolation peep variants, without differential reward (i.e., no punishment for incorrect responses). All three of the monkeys demonstrated spontaneous discrimination of several variants, some of which were nearly identical in several physical features. By varying loudness, and using calls of differing durations, we established that these spontaneous discriminations were not based on simple acoustic features shared with the original training pair, but represented complex feature recognition of subtle variations in acoustic structure of this call type. In a second study, models of isolation peeps were synthesized with a PDP-12 computer, permitting control of specific components of acoustic structure. Using a paradigm similar to the first study, four monkeys were trained to discriminate between a natural isolation peep and an artifically synthesized model. However, all four failed to reliably discriminate between two artificial models. These results are consistent with other studies showing that monkeys do not attend closely to artificial acoustic stimuli.

3) Sound spectrographic analysis: Using the Voiceprint sound spectrograph, analyses have continued to assess the transfer of information by vocalizations. Extensive analyses of one pair of squirrel monkeys has shown that the acoustic structure of certain call types are distinctive for each individual. Distinctive differences are reflected in several attributes of vocal structure: call duration, number of syllables, and complexity of syllable structure, as well as in more subtle aspects of acoustic structure.

These findings suggest that the physical nature of vocalizations serves as a "vocal signature" for the individual monkey.

Significance to Biomedical Research and the Program of the Institute: These findings have provided further substantiation of earlier results in this project that squirrel monkeys possess a complex acoustic communication system, that natural variations in the acoustic structure of certain vocalizations are differentiated by the animal, and that many neurons in the auditory cortex possess attributes necessary for the perception of these vocal differences.

Proposed Course: The work to date in this project has produced a substantial description of the encoding of vocalizations by neurons in the primate auditory cortex, and suggested some behavioral correlates of this neural processing. Future work may now begin to deal with other related questions: 1) the role of early experience and inheritance in the establishment of neuronal specificity to vocalizations; 2) the functional relationships of other brain systems (especially the frontal-limbic system) to the auditory cortex; 3) the relationship between chemical specificity (as defined by differential sensitivity to various transmitter substances) and sensory specificity (as defined by differential specificity to vocalizations).

Honors and Awards: None

Publications:

1. Newman, J. D. and Wollberg, Z. Multiple coding of species-specific vocalizations in the auditory cortex of squirrel monkeys. Brain Research 54: 287-304, 1973.

2. Funkenstein, H. and Winter, P.: Responses to acoustic stimuli of units in the auditory cortex of awake squirrel monkeys. Exptl. Brain Res., in press.

3. Winter, P. and Funkenstein, H.: The effect of species-specific vocalization on the discharge of auditory cortical cells in the awake squirrel monkey (Saimiri sciureus). Exptl. Brain Res., in press.

4. Evans, E. F. and Nelson, P. G.: On the functional relationship between the dorsal and ventral divisions of the cochlear nucleus of the cat. Exptl. Brain Res., in press.

5. Evans, E. F. and Nelson, P. G.: The responses of single neurons in the cochlear nucleus of the cat as a function of their location and the anaesthetic state. Exptl. Brain Res., in press.

Serial No. HD-BB5
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Neurobiologic studies of neuronal and other cell types in cell culture.

Previous Serial Number: Same

Principal Investigators: Phillip G. Nelson, M.D., Ph.D.
Earl L. Giller, M.D., Ph.D.

Other Investigators: Harris R. Fisk, M.D., Ph.D.

Cooperating Units: Department of Biology, Yale University, New Haven, Connecticut
Laboratory of Biochemical Genetics, National Heart and Lung Institute, NIH
Laboratory of Molecular Biology, The Weizmann Institute, Rehovot, Israel

Man Years:

Total:	7.5
Professional:	3.5
Others:	4.0

Project Description:

Objectives: To describe neurobiologically interesting properties of normal and continuous lines of cells in tissue culture, to elucidate the molecular basis for these properties and to analyze the control mechanisms regulating their expression. In particular, the electrophysiologic and metabolic interactions between different cell types are of great interest.

Methods Employed: Intracellular microelectrode recording and iontophoretic application of materials to the exterior and interior of cultured cells are used in analyzing cell function. Subcellular fractionation and gel electrophoretic separation of the protein constituents of the various subcellular fractions, in particular the surface membranes have been continued. A variety of culture media have been studied for their capacity to support neuronal survival and differentiation.

Major Findings: Mouse cerebellum has been extensively studied in other laboratories from the standpoint of maturational timetable for various cell types and the characteristics of a number of neurologic mutants which involve

the cerebellum. We have found that cell cultures prepared by dissociating the fetal mouse cerebellum produce networks of electrically active and synaptically interacting neurons. Both excitatory and inhibitory postsynaptic potentials have been recorded and vigorous rhythmic, synaptically mediated patterns of electrical impulses are generated within these networks. A variety of morphologically and electrophysiologically defined cell types were seen so that some substantial representation of the neuronal constituents of the cerebellum should be available for study in vitro.

A detailed study of the culture conditions needed to optimize mouse spinal cord and muscle culture has been made as a prerequisite for investigating the biochemical consequences of the establishment of synaptic interactions between these cells. Variation in the type of serum in the medium and the timing of the use of different antimetabolites have been shown to be important in producing the best survival and differentiation of both nerve and muscle.

Since chick spinal cord and muscle have been shown to be favorable preparations for study in cell cultures, we have used this material for an initial study of a genetically determined disease, that of muscular dystrophy. Both dystrophic muscle and nerve develop well in cell culture and both normal and dystrophic nerve establish plentiful neuromuscular junctions with dystrophic muscle. The incidence of such synaptic connection appeared higher than in normal cultures as did the incidence of electrical coupling between cells in these pathological cultures. The significance of these findings remains to be investigated, but it is an attractive hypothesis that the disease is in part due to a defect in the trophic function that is involved in preventing multiple innervation of muscle.

Calcium ions may play an important role in coupling surface membrane electrical events to cytoplasmic metabolic processes (see projects of Drs. Gainer and Henkart). We have found (in collaboration with Dr. Ilan Spector) that in some clones of neuroblastoma cells a significant proportion (10-20%) of the ionic currents which flow during the action potential are resistant to tetrodotoxin and are blocked by cobalt which indicates that they are calcium currents. The active electrical response in fibroblasts (L-cells) elicited by mechanical, electrical and chemical stimuli also involves calcium ions, for intracellular injection of calcium ions elicits the electrical response. Both these cell lines and products of their hybridization represent favorable material for the study of transmembrane and intracyloplasmic calcium movements and the metabolic and structural consequences of such calcium movement.

Four to five fold enrichment for surface membranes as measured by radioiodine labelling of these membranes has been achieved by differential centrifugation. Distinctive patterns of membrane proteins in L-cells and one clone of neuroblastoma cells have been shown by disc gel electrophoresis. The resolution of the system is good and its application to a more extensive range of material is indicated.

Significance to Biomedical Research and the Program of the Institute: From a general standpoint, the use of well-defined tissue culture systems is playing an increasingly important role in analyzing neurobiologic problems in

cellular and molecular terms. Neuronal differentiation and the development and maintenance of complex cell-cell relationships are crucial for normal system function and these processes can be studied in detail in the culture systems. Specifically, the relationship of surface membrane structure (protein composition) and function (action potential generation and Ca++ fluxes) to cell metabolism and intercellular interactions are of significance to these broader areas. The direct study of various neuropathologic states with tissue culture models is a promising approach but the more general understanding of normal developmental mechanisms is of probably greater importance.

Proposed Course: Biochemical and morphological studies of the interactions between different classes of neural cells in culture has begun and will be continued. What are the biochemical consequences when synaptic connections are formed in the different preparation? Are neural transmitter related enzyme or other cell constituents induced or repressed? If so what is the mechanism? Further histochemical and microchemical characterization of the cell types occurring in the different normal cultures will be made.

Clonal analysis of the control of surface membrane properties such as calcium permeability mechanisms and chemosensitivity will be continued. The relationship between these properties and cell metabolism will be studied in neuroblastoma and L-cells. We will continue the analysis of membrane composition on a broader range of cell lines and attempt to relate these analyses to physiologic function in these lines.

Honors and Awards: None

Publications:

1. Peacock, J. H. and Nelson, P. G.: Chemosensitivity of mouse neuroblastoma cells in vitro. J. Neurobiol., in press.

2. Peacock, J. H., McMorris, F. A. and Nelson, P. G.: Electrical excitability and chemosensitivity of mouse neuroblastoma X mouse or human fibroblast hybrids. Exptl. Cell Res., in press.

3. Nelson, P. G. and Peacock, J. H.: Electrical activity in dissociated cell cultures from fetal mouse cerebellum. Brain Res., in press.

4. Peacock, J. H., Nelson, P. G. and Goldstone, M. W.: Electrophysiologic study of cultured neurons dissociated from spinal cords and dorsal root ganglia of fetal mice. Develop. Biol. 30: 137-152, 1973.

5. Peacock, J. H. and Nelson, P. G.: Synaptogenesis in cell cultures of dystrophic chick neurons and myotubes. J. Neurol. Neurosurg. Psychiat., in press.

6. Nelson, P. G. and Peacock, J. H.: Acetylcholine responses in L-cells. Science 177: 1005-1007, 1972.

7. Nelson, P. G. and Peacock, J. H.: Transmission of an active electrical response between fibroblasts (L-cells) in cell culture. J. Gen. Physiol., in press.

Serial No. HD-BB6
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Synapse formation between nerve and muscle cells in tissue culture.

Previous Serial Number: Same

Principal Investigator: Gerald D. Fischbach, M.D.

Other Investigators: Phillip Nelson, M.D., Ph.D.
Maryanna Henkart, Ph.D.
Stephen A. Cohen, M.D.
John Whysner, M.D., Ph.D.
Anthony C. Breuer, M.D.

Cooperating Units: Drs. V. T. Marchesi and B. Hirani, National Institute of Arthritis and Metabolic Diseases.

Man Years:

Total:	4.5
Professional:	4.0
Others:	0.5

Project Description:

Objectives: Our long-term objective remains the study of neuron and muscle cell differentiation and synapse formation in cell culture. We plan to continue study of the sequence of events during nerve-muscle junction formation, of factors that regulate postsynaptic membrane chemoreceptors, of the degree and mechanism of specificity in interneuronal synapse formation and of the development of different types of electrically excitable membranes. In each of these areas, ultrastructural and biochemical correlates of electrophysiologically defined phenomena will be sought.

Methods Employed: Conventional cell culture and intracellular microelectrode techniques remain the backbone of our experimental approach. Relatively purified cultures (muscle and nerve cells) have been obtained by the use of drugs that kill dividing cells and a "low growth" culture media.

Autoradiographic and electron microscopic techniques have been tailored to the cell culture system. Time-lapse cine studies of growing neurites and muscle cells are underway. Histologic techniques include silver, acetylcholinesterase and methylene blue stains. With Drs. Marchesi and Hirani, we have experimented with immuno-ferritin techniques.

Major Findings: Innervation does not change the general level of muscle membrane ACh sensitivity but muscle activity does. Fibers chronically stimulated through large electrodes implanted in the culture dish are less sensitive than unstimulated fibers and less than one-tenth as sensitive as completely inactive fibers (grown in the presence of tetrodotoxin or lidocaine). Changes are nearly complete within 24 hours.

Several specializations of the surface membrane have been identified by thin section and scanning electron microscopy (by M. Henkart) which may explain areas of increased ACh sensitivity. Coarse membrane folds, microvilli and an extensive tubulo-vesicular network that communicates with the extracellular space are likely candidates. Attempts to directly visualize receptor sites with α-bungarotoxin-ferritin conjugates have met with some success. Small clusters of ferritin (clusters of receptors) have been identified by freeze etch and transmission EM techniques. Time-lapse cine studies have shown that muscle nuclei move long distances (up to 300 μ) within muscle fibers at rates as fast as 40 μ/hr. This may explain some of the exceptions in the correlation between muscle nuclei and hot spots.

ACh receptors of cultured muscle cells bind α-bungarotoxin in the same manner as receptors in other cells. Toxin binding sites increase as myoblasts fuse with one another and reach a plateau after 4-6 days. The initial rate of binding can be reduced to less than 5% of control by pre-incubation with d-Tubocurare but toxin eventually overcomes the curare competition. The binding, which is apparently irreversible, is not altered when the muscle membrane, is depolarized by high K^+ solutions. The time course of binding is closely paralleled by decline in ACh sensitivity. There is no "lag" in depression of sensitivity so it is unlikely that a large pool of "spare" receptors exist.

Functional nerve-muscle contacts have been identified as early as 60 hours after addition of spinal cord cells to muscle cultures. Attempts to demonstrate coupling at these early contacts by injection of the dye proscion yellow into muscle fibers have, so far, been negative.

Significance to Biomedical Research and the Program of the Institute: The mechanism of synapse formation is a basic question in embryology. Formation and interuption of synaptic coupling between nerve and various nerve and muscle cells has profound structural and metabolic consequences. The tissue culture technique offers considerable hope for analyzing this striking biologic phenomenon. Modification of synaptic function by changes in activity pattern would help in understanding mechanism of neuronal plasticity or learning. Before asking the relevant biochemical questions, the basic electrophysiological phenomena must be understood. Tissue culture has provided a convenient system in this regard.

Proposed Course: We plan to focus on early nerve-muscle synapses to determine the relation between nerve terminals, hot spots and muscle nuclei and to further search for direct electrical coupling. Toxin-ferritin conjugates will be used to determine the distribution of receptors over the various membrane specializations described above. The purity and stability of the conjugates must be determined. In addition, intracellular sites of receptor

synthesis will be sought by applying conjugates to protein embedded sections of muscle cells. The relation between activity and surface ACh receptors will be further analyzed after uncoupling membrane excitation from subsequent contraction. Combined, D_2O and cobalt treatment (to prevent contraction) has been the best means so far. The distribution of sensitivity over stimulated innervated fibers will be determined to see if nerve terminals maintain hot spots. The super-sensitivity of inactive fibers will be studied to determine if it is due to new synthesis of receptors or to uncovering of pre-existing sites.

Honors and Awards: None

Publications:

1. Fischbach, G. D. and Cohen, S. A.: The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. Develop. Biol., in press.

2. Cohen, S. A. and Fischbach, G. D.: Regulation of muscle acetylcholine sensitivity by muscle activity in cell culture. Science, in press.

3. Fischbach, G. D., Fambrough, D. and Nelson, P. G.: A Symposium on neuron and muscle cell cultures. Fed. Proc., in press.

4. Fischbach, G. D., Henkart, M., Cohen, S. A., Whysner, J. A. and Breuer, A.: Neuromuscular junction formation in culture. 26th Symposium of Society of General Physiologists, Woods Hole, Massachusetts, 1972, in press.

Serial No. HD-BB8
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Correlative neurochemical and electrophysiological analyses of individual, identifiable neurons in Molluscan nervous systems.

Previous Serial Number: Same

Principal Investigator: Harold Gainer, Ph.D.

Other Investigators: Jeffery L. Barker, M.D.

Cooperating Units: Thomas Smith, M.D., Laboratory of Neurophysiology, NINDS
Ichiji Tasaki, M.D., Laboratory of Neurobiology, NIMH and Marine Biological Laboratory, Woods Hole, Mass.
Maryanna Henkart, Ph.D., Behavioral Biology Branch, NICHD
Milton Brightman, Ph.D. and Lisa Prescott, M.D. Laboratory of Neuropathology, NINDS
Raymond Lasek, Ph.D., Case-Western Reserve Univ., Department of Anatomy, School of Medicine and Marine Biological Laboratory, Woods Hole, Mass.

Man Years:

Total:	2.5
Professional:	2.0
Others:	0.5

Project Description:

Objectives: To study the mechanisms by which the physiological activities of identifiable neurons are coupled to their macromolecular synthesis patterns.

Methods Employed: Several methods for the separation and analysis of proteins synthesized by single neurons have been developed in this laboratory. These include dissection of the individual cells from the ganglion under a low-power microscope with the use of fine needles as well as mechanical homogenization of the cells and chemical solubilization of their proteins. The analytical tools include (1) general electrophoresis of proteins in which their separation is based on molecular weight and charge, (2) polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulfate (separation based on molecular weight only) and (3) isoelectric focusing of proteins

(separation based on charge only). All of these separation techniques have been developed and used on both the macro and the micro scale, and together with radioisotopes of amino-acids and other labeled precursors, considerable information has been gathered about the protein metabolism of single neurons. Other "single-cell" chemical methodologies being developed in our laboratory include (1) ultracentrifugation of membranes and cytoplasmic contents, (2) methods in lipid and liproprotein biochemistry, (3) nucleotide biochemical techniques, and (4) intracellular isotope injection techniques for axoplasmic transport studies.

Two experimental preparations are being utilized for these studies. The isolated brain of the land snail, Otala lactea, and the abdominal ganglion of the sea hare, Aplysia californica. Both nervous systems contain large, identifiable neurons which are appropriate for combined electrophysiological-neurochemical studies. Conventional electrophysiological recording and stimulating methods are also employed in these studies. Some of these methods are (1) intracellular recording from individual, identified cells in molluscan ganglia, (2) stimulation techniques for the purpose of studying synaptic transmission, (3) manipulation of the ionic environment for the purpose of studying membrane potentials and their ionic bases and (4) voltage-clamp techniques for the purpose of studying the ionic currents involved in membrane potential changes.

In addition, the giant axon of the squid has been intracellularly perfused with protein radiodination solutions (i.e., lactoperoxidase techniques) in a search for functionally significant nerve membrane proteins.

Major Findings: Extensive investigations of five identified neurons in Otala lactea and eight identified neurons in Aplysia californica revealed that individual neurons had distinct and unique patterns of protein synthesis (Gainer and Wollberg, in preparation). In particular, one class of neurons appeared to exhibit sufficient plasticity to warrant a study of the effects of electrical and synaptic activity on their synthesis patterns. Consequently, we have investigated whether physiological input to a nerve cell can selectively and significantly influence the biochemical processes of that cell. For this purpose we have chosen to study the effects of synaptic stimulation on the regulation of protein synthesis in an identified neurosecretory cell found in the mollusca, Aplysia californica. The bursting pacemaker neuron, R_{15}, in the abdominal ganglion of Aplysia californica exhibits a specific pattern of protein synthesis when incubated in ^{3}H-Leucine. SDS-polyacrylamide gel electrophoresis shows that 20-25% of the de novo synthesized proteins of the cell is accounted for by a single molecular weight class (around 12,000 daltons). Stimulation of the branchial nerve for 5 hours at a rate of 5/sec, hyperpolarized the cell and blocked all spontaneous spike activity. Comparisons of the protein synthesis patterns of synaptically inhibited neurons and spontaneously active control neurons showed that synaptic inhibition produced a selective 30% decrease in the synthesis of the 12,000 dalton peak. The putative inhibitory transmitter, dopamine, produced the same selective inhibition when added to the bathing medium. Depolarization of the cell by increasing the potassium ion concentration of the medium, selectively stimulated the synthesis of the 12,000 dalton protein by 50-70%. Thus, the rate of synthesis of the 12,000 dalton protein in R_{15} may

be regulated by the membrane potential of the cell, which in turn is under control of a dopaminergic inhibitory synapse. Studies are presently under way to elucidate the mechanisms underlying this regulation phenomenon.

Studies on the protein synthesis of the giant axon of the Squid (in collaboration with Dr. R. Lasek, at the Marine Biological Laboratory, Woods Hole, Massachusetts) have led to the observations that the axon's newly synthesized protein has its origin in the Schwann cell layer immediately surrounding the axon. We have considerable evidence for the intercellular transfer of these proteins (ranging from 0.5 million to < 12,000 daltons, in preparation) and now are studying the regulation mechanisms underlying these glial-neuronal interactions. Other studies on the squid axon (with Dr. Tasaki) have revealed a small, labile protein on the inner surface of the axon membrane which is affected by depolarization (see Gainer, et al, Biol. Bull., 1972).

Our electrophysiological studies of the above-mentioned neurosecretory cells have revealed that divalent cations play a major and important regulatory role in both the bursting pacemaker potential activity of these cells and their seasonal modulation. As part of this project, we have concluded from our intracellular studies that bursting pacemaker potential activity is due predominantly to a time- and voltage-dependent potassium conductance, modulated by divalent cations and superimposed upon a constant sodium conductance. In collaboration with Dr. T. Smith, we are now applying the voltage clamp technique to an investigation of the ionic currents involved at various phases of the bursting pacemaker potential cycle. Our results indicate that the current flow during the depolarizing phase of the cycle is predominantly inward, being predominantly dependent on both external sodium and divalent cation concentration. This current (and the bursting pacemaker potential activity) can be antagonized by cobalt applied externally. The flow of current at the start of the hyperpolarizing phase of the cycle is mainly outward and can be antagonized by both barium and tetraethylammonium. From these initial findings we have concluded that bursting pacemaker potential activity is primarily dependent on two membrane properties: (1) a voltage dependent inward divalent (calcium and magnesium) - monovalent cation (sodium) current closely coupled to (2) a voltage-dependent outward potassium current. These studies have demonstrated an important regulatory role for divalent cations both in the seasonal modulation of the neurosecretory cell's rhythm and the bursting pacemaker potential itself. We plan to study the effects of divalent cations on the protein synthesis patterns of the neurosecretory cell in an attempt to better understand how the snail controls neurosecretion during aestivation.

In collaboration with Dr. M. Henkart (electron microscopy) and Drs. M. Brightman and Prescott (freeze fracture and etching), we have initiated a study correlating the morphological changes in these neurosecretory neurons with their electrical, metabolic, and secretory activity.

Significance to Biomedical Research and the Program of the Institute: The approach described above is specifically designed to correlate relevant biochemical changes taking place in the nervous system with its specific functional activity. The heterogeneity of nervous tissue makes the most

direct approach to this problem a desirable one. In this approach individual identifiable neurons can be studied electrically and biochemically thereby providing a direct correlation between these events - and it is hoped ultimately an insight into what membrane phenomena in neurons lead to long-term intraneuronal biochemical changes. The molluscan nervous system represents an excellent model for not only excitation-metabolic coupling studies, but also as a model of hypothalamic regulatory systems in mammals. The neuron upon which we are focusing our efforts, is modulated by dopaminergic synaptic input, and appears to receive long-term negative feedback via circulatory steroids, and positive feedback by small peptides. Thus, in this single neuron, there resides a wide range of phenomenology characteristic of hypothalamic nuclei, which are amenable for detailed study in this model system. We believe that such model systems are essential for the analysis of regulatory mechanisms in neurons and developing nervous systems.

Proposed Course: In view of our current findings that protein synthesis can be selectively modulated by synaptic input, we intend to study the membrane mechanisms which are coupled into the intracellular biochemical control systems. After investigation of the ionic mechanism(s) underlying the initial step at the membrane level, we plan to study the other steps involved in the transduction of the signal and regulation of protein synthesis. Does the initial signal act at a particular reaction in the protein synthesis pathway (e.g., at the transcriptional or at the translational level or both)? Are there secondary or tertiary messengers involved in transduction? A major effort will be made to determine which sub-cellular organelles are involved in the activity-metabolic coupling processes, and to characterize the functional morphology of the cell's ultrastructure via a variety of techniques (e.g., electron microscopy and autoradiography, freeze fracture, etc.).

Honors and Awards: None

Publications:

1. Gainer, H.: Isoelectric focusing at the 10^{-10} to 10^{-9} gram level. Anal. Biochem. 51: 646-650, 1973.

2. Gainer, H., Carbone, E., Singer, I., Sisco, K. and Tasaki, I. Identification of some membrane protein subunits obtained from the squid giant axon. Biol. Bull. 143: 462-463, 1972.

3. Colburn, T. R., Wollberg, Z. and Gainer, H.: A logarithmic display of interspike-interval for long-term, patterned neural activity. Medical and Biological Engineering, in press.

4. Barker, J. L. and Levitan, H.: The antagonism between salicylate-induced and pH-induced changes in the membrane conductance of Molluscan neurons. Biochem. et Biophysica Acta 274: 638-643, 1972.

5. Levitan, H. and Barker, J. L.: Salicylate: A structure activity study of its effects on membrane permeability. Science 176: 1423-1425, 1972.

6. Levitan, H. and Barker, J. L.: Membrane permeability: Cation selectivity reversibly altered by salicylate. Science 178: 63-64, 1972.

7. Levitan, H. and Barker, J. L.: Effect of non-narcotic analgesics on membrane permeability of Molluscan neurons. Nature New Biology 239: 55-57, 1972.

Serial No. HD-BB9
1. Behavioral Biology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: A correlated study of the fine structure and the function of the endoplasmic reticulum (ER) of neurons.

Previous Serial Number: New project.

Principal Investigator: Maryanna Henkart, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.8
Professional:	0.8
Others:	1.0

Project Description:

Objectives: To develop and apply methods for demonstration with the electron microscope of sites of Ca binding or accumulation in order to explore the possibility that certain physiological and/or metabolic functions in neurons are regulated in coordination with membrane activity via Ca entry or release of Ca from internal stores.

Methods Employed: Two systems are being studied: 1) the squid giant axon and 2) identified neurons of Aplysia (R_2 and R_{15}). The fine structure of the endoplasmic reticulum is being analysed in these preparations using serial sectioning and section tilting techniques. The identification of Ca-accumulation sites is being carried out with techniques involving fixation in the presence of various concentrations of divalent ions (Ca, Mg), substitution of more electron-opaque divalent ions (Sr, Co, La), and fixation in buffer solutions that should precipitate divalent ions of interest (PO_4). The tissues are fixed under various physiological conditions produced by alteration of the ionic compositions of the physiological saline bathing the living material (e.g. depolarized by increased external K^+). The changes of morphology of the ER under these various conditions are analyzed.

Major Findings: The fine structure of the endoplasmic reticulum of the squid giant axon changes under conditions in which the divalent ion composition of the external solutions are altered. The percent of surface membrane forming junctions with cisternae of the endoplasmic reticulum varies with the divalent ion concentration and the divalent ion species in the external solutions.

Sr is visible as dense deposits in cisternae of the endoplasmic reticulum. When the axon is depolarized the ER swells in the presence of increased external divalent ion concentration. The swelling produced in the presence of a given concentration of Sr or Ca is greater than that produced in the presence of the same concentration of Mg. Some similar changes have been observed in the ER of Aplysia neurons.

Significance to Biomedical Research and the Program of the Institute: The observations so far suggest an analogy between the endoplasmic reticulum of neurons and the sarcoplasmic reticulum of muscle which is known to function as a Ca-sequestering and releasing system. In muscle the Ca release is coupled with membrane potential changes, and increased internal free Ca triggers muscle contraction and controls the activity of at least one enzyme, phosphorylase b-kinase that results in the mobilization of glucose from glycogen, providing an increase in energy available to support the muscle activity. If the analogy holds for nerve cells it suggests that the ER may be involved in coupling the electrical activity of the surface membrane to intracellular activities, the coupling being mediated by Ca released from the ER. If this is the case it could represent a critical control mechanism for a number of neuronal activities - possibly including growth and differentiation.

Proposed Course: The function of the ER in controlling the intracellular Ca concentration in neurons must be further substantiated. Changes in the ER in electrically and synaptically stimulated neurons must be studied. In collaboration with Drs. Gainer and Barker the possibility that activity is coupled to synthesis of specific proteins via Ca will be investigated. In collaboration with Dr. A. Breuer we intend to test the effect of altered divalent ion compositions on intra-axonal movement of particles directly observed with Nomarski optics. If alterations of external divalent ion composition or concentration alter the rate or direction of particle movement the material will be fixed and examined with the electron microscope to ascess changes in configuration of the ER or other organelles that may be involved in particle transport.

Honors and Awards: None

Publications: None

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Laboratory of Molecular Genetics

The Laboratory of Molecular Genetics conducts research directed toward understanding the molecular processes involved in the transmission of genetic information from generation to generation and in the regulated expression of this genetic information in cells during growth and differentiation. A variety of model systems are under investigation in the laboratory, ranging from bacterial and animal viruses to complex vertebrate regulatory responses involving the synthesis of specific antibodies and the development of specific organ systems. Taken together, the laboratory joins active workers in the areas of genetics, biochemistry, immunology, virology and cellular biology to bring their skills to bear on problems of mutual interest. During the past year, the Laboratory of Molecular Genetics has, for the first time, had an opportunity to join together in a single building. At present, the laboratory is comprised of seven research groups. Their activities are described in the phylogenetic order of the biologic systems with which their research is primarily concerned.

A. The process of integration and excision of the phage λ has been shown to occur at a number of sites on the E. coli chromosome when the normal attachment site from λ is removed. This integration has been shown to occur at specific sites in the E. coli chromosome (integration is nonrandom). The process of integration and excision from these sites requires the same phage gene necessary for normal integration and excision. This process is not influenced by the recombination systems controlled by the bacterial host chromosome. The process by which the λ chromosome is organized into the phage particle has been investigated also. When the normal processing mechanism is interrupted, phage particles appear to accumulate a "headful" of λ DNA, and processing, that is cutting, of viral genome occurs by alternative mechanisms. These investigators have also shown that host chromosomal material can be incorporated into the λ genome. This observation provides the initial evidence that phage λ can be used as a generalized transducing phage. In addition, the genes necessary for the formation of the viral particle have been examined and evidence suggests that these proteins are produced in a coordinate fashion suggesting the existence of controls at some post-transcriptional level. Taken together, these results give a much clearer picture of the process of viral insertion and excision and the elements necessary for the packaging of the viral chromosome in immature virus particles.

B. Factors which influence the initiation and termination of genetic transcription have been further investigated in terms of regulatory proteins and ribonucleases. Specifically, an extremely interesting ribonuclease, ribonuclease H, has been isolated from a number of sources. This enzyme specifically degrades RNA which forms a duplex with a strand of complementary DNA. Inasmuch as RNA transcription is thought to be intimately related to gene replication, this

enzyme may have an important role in the process of chromosomal replication. Ribonuclease H has been shown to be an integral part of the avian myeloblastosis reverse transcriptase enzyme. This enzyme has also been isolated from E. coli, and a determined effort has been made to correlate it with one of the temperature sensitive mutants defective in DNA replication. None of these seem affected. In addition, ribonuclease H activity has been found in association with DNA polymerase I of E. coli, but not with the DNA polymerase which can be isolated after the infection of E. coli with bacteriophage T4. In the course of this work, a factor has been identified from E. coli which depresses the level of RNA synthesis and which may be related to the physiologic termination of mRNA synthesis in E. coli.

C. Studies on the integrative control of macromolecular synthesis have resulted in the development of a number of interesting observations. Two unusual guanine nucleotides, ppGpp and pppGpp, have been thought to function in the regulation of protein and nucleic acid biosynthesis in bacterial cells. This year, purified cell-free systems have been devised which indicate that a portion of the protein synthetic apparatus of the cell is directly involved in the biosynthesis of these nucleotides. Specifically, the synthesis of ppGpp requires highly purified ribosomes, transfer and messenger RNA as well as appropriate phosphate accepting molecules. The fact that these nucleotides are synthesized by ribosomes "at rest" correlates well with the physiologic observation that these nucleotides accumulate when protein synthesis is inhibited. Studies on the mechanism of phosphorylation suggest that this occurs by pyrophosphorylation. In addition, the protein synthetic system can be used to prepare large amounts of these compounds for further study.

These guanine nucleotides have been shown specifically to stimulate several bacterial operons which can be transcribed and translated in a cell-free system. Evidence suggests that this regulation is affecting the transcription reaction, namely RNA polymerase activity. Additional experiments have shown that the guanine nucleoside ppGpp can interact with the elongation factor Tu, but that it will not substitute for pppG in protein biosynthesis. In contrast, pppGpp can substitute for pppG in several of the protein biosynthesis reactions.

Taken together, these results suggest a close metabolic connection between the processes of translation and transcription, possibly involving mediation by ppGpp or pppGpp.

D. The process of regulated gene expression has been investigated in viral systems as well. The simplest of these has been the human adenovirus, a relatively small DNA-containing virus which serves as a model for the regulated expression of a group of genes in higher organisms. Work with this system began in September, 1972, and results are, therefore, preliminary at this point. The basic strategy involved has been to isolate specific adenovirus mRNAs and to correlate these mRNAs with specific viral proteins using cell-free systems to

identify products. The specific mRNAs will then be used to establish a genetic and regulatory map of the adenovirus genome. Thus far, adenovirus mRNA has been isolated using oligo-dT-cellulose affinity chromatography. This mRNA has been translated in the Krebs ascites II cell-free system, giving rise to a unique and reproducible pattern of bands in polyacrylamide gels. The analysis of these products and their correlation with specific viral proteins is underway.

E. Certain aspects of the process of differentiation have been studied using embryonic chick lens fibers in vivo and in tissue culture. These studies have been directed towards the description of the process of normal differentiation of lens fibers and towards understanding these regulatory mechanisms at the molecular level. During the past year, these workers have shown that the terminal phases of lens fiber differentiation can occur in tissue culture. These results correlate well with previous studies of this group indicating that the initial stages of this process can also occur in this tissue culture system. This observation has been coupled with the finding that insulin can substitute for serum in the culture medium stimulating the assembly of microtubules and the elongation of the lens fiber cells. In contrast to serum, insulin does not promote the de novo synthesis of delta crystallin. Additional studies have been directed toward the isolation and translation of delta crystallin mRNA. mRNA has been isolated from purified lens fibers and eluted from oligo-dT-cellulose. This material has been shown to direct cell-free protein synthesis in the Krebs ascites system. Studies are currently underway to characterize the product and to identify it as chick lens delta crystallin. If these studies are successful, the mRNA will be further purified and transcribed using the avian myeloblastosis reverse transcriptase. This cDNA can then be used in studies viewing the regulated appearance of delta crystallin mRNA in tissue culture material.

F. Studies directed towards understanding the regulated expression of the globin and immunoglobulin genes have permitted workers to draw several important conclusions. These studies have indicated that the reverse transcript of purified globin message can be used as a highly specific and sensitive probe for quantitating specific globin genes and mRNA. These studies indicate that the number of genes corresponding to globin in a variety of avian and mammalian tissues is rather small, 3 to 5 globin genes per diploid genome, and is not amplified during the process of differentiation. With respect to globin genes, the gene amplification hypothesis appears to be ruled out. Further studies have indicated that the process of differentiation can be studied using tissue culture cells. These studies suggest that the transcription of the globin genes represents a rate limiting step in the process of differentiation of these cells. Globin message has been quantitated and rises from an undetectable amount to 3,000 to 6,000 molecules per cell in the fully differentiated state.

Further studies of the immunoglobulin light chain message suggest that it is a poly(A) containing mRNA which directs the synthesis of both the constant and variable regions of the immunoglobulin light chain. This mRNA can also be

transcribed using the reverse transcriptase. Hybridization analyses using this cDNA suggest that the mRNA preparations, in contrast to those of the globin mRNA, are not yet pure. Further studies indicate that there is a sequence of cDNA present which corresponds to a highly reiterated component of the mouse genome. The implications of these data are being considered in the light of models which can be used to explain the diversity of antibody molecules.

The techniques developed in the course of these studies have had direct implications for examining the pathophysiology of thalassemia, an inherited human anemia. A number of laboratories have been using the reverse transcript of human alpha and beta globin mRNA to quantitate the respective globin mRNAs in reticulocytes of infected individuals.

G. Antibody biosynthesis has also been studied at the cellular level. Experiments have been carried out to clarify the role of specific cell types in the induced immune response. These have demonstrated that a derivative of cyclic AMP can amplify the number of B type cells responding to specific immunization. In addition, studies which indicate certain antigens have an initial suppressive T cell which may regulate the activity and numbers of B cells involved in a specific immune response. One extremely important observation made by these workers relates to the in vitro production of antibodies by immunocytes. It seems that a number of plaques noted using the plaque-forming center assay with rabbit spleen cell cultures may be due to pre-formed background antibody which is present in homologous rabbit serum used in all these assays. This observation has great importance for all studies of in vitro antibody formation. It will doubtless cause considerable re-thinking of experiments carried out in this area.

Preliminary studies have suggested that the antibody required to develop and visualize 7S IgG secreting cells is itself a high affinity IgG antibody with major anti-Fc activity. In addition, an antiserum containing a strong suppressive activity directed towards 19S IgM placque-forming cells has also been discovered. The active agent appears to be a high affinity IgG with a major anti-Fab activity. These reagents are now being prepared using isolated immunoglobulin fractions as antigens. As such, they may have relevance to the mechanism of initiation and suppression of the respective immune responses.

The Laboratory of Molecular Genetics has past its first year in our new Building 6 location. The new groups now located adjacent one another have created an impressive and exciting scientific atmosphere. A great deal of productive exchange between working groups has occurred. The organizational phase of the laboratory's activities having passed, we now look forward to the further development and expansion of the younger research groups. As a number of us are interested in taking advantage of the electron microscope for purposes of genetic mapping, we hope to attract an excellent scientist who is an expert in this area during the coming year.

Serial No. HD-LB16
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Peoject Report
July 1, 1972 through June 30, 1973

Project Title: Control Mechanism in Temperate Bacteriophage λ

I. The control of normal phage integration and excision
II. Secondary pathways of phage integration and excision
III. The physiology of abnormally excised phage
IV. Packaging of viral and bacterial DNA by bacteriophage λ.

Previous Serial Number: Same

Principal Investigator: R. Weisberg

Other Investigators: N. Sternberg

Cooperating Units: Laboratory of Molecular Biology, Section on Biochemical Genetics, NCI

Dr. Ronald Luftig
Department of Microbiology, Duke University, Durham, North Carolina
Dr. Kazunori Shimada
Department of Biological Sciences, Stanford University, Palo Alto, California

Man Years

Total: 24/12
Professional: 24/12
Others: 0

Project Description:

Objectives: To understand the mechanism of the insertion of virus DNA into and its excision from the chromosome of its host; to understand the control the phage exerts over the synthesis of proteins which are required for these processes; to study the nature of abnormal phage insertion and excision with the aims of understanding the normal process, on the one hand, and of determining the feasibility of using abnormal insertion as a tool for biochemical analysis of bacterial gene products on the other; to analyze the mechanism of

conversion of the virus nucleic acid to a mature, infectious virus particle.

Methods Employed: The organisms of study are bacteriophage λ, other genetically related phages, and their host, the bacterium E. coli. Genetic techniques are being used to obtain cells which carry virus DNA inserted at abnormal chromosomal sites; from such abnormal lysogens, we obtain novel types of transducing phage lines (phage strains whose chromosomes contain DNA of bacterial origin). Phage mutants with specific chromosomal aberrations are being used to characterize the activity and stability of the factors which govern insertion and excision. Temperature sensitive control mutants are being used to study the regulation of the synthesis of these factors. Density gradient analysis and electron-microscopic visualization of DNA are being used to determine the structure of the chromosome of a newly isolated phage variant. A bacterial mutant which is defective in one of the steps of virus morphogenesis is being used to characterize the cellular structures required for viral development. Likewise, morphogenesis-defective phage mutants are being used to determine the sequence of steps involved in the synthesis of the λ head.

Major Findings: (1) In the course of lysogenization by phage λ, the viral DNA is inserted into the bacterial chromosome at a specific chromosomal site called the attachment site. We have shown that insertion still occurs, although at reduced frequency, in mutant bacteria which have lost the attachment site. Such abnormal insertion can occur at many different chromosomal locations, although it otherwise resembles normal insertion. Ocassionally, abnormal insertion of the virus DNA occurs within a bacterial gene, thereby inactivating the function of that gene. Not all genes are equally likely to be inactivated: the frequency of inactivation for different genes varies over a greater than 10^6-fold range. This highly nonrandom pattern of inactivation suggests that abnormal insertion occurs preferentially at a limited number of sites. We have confirmed this hypothesis by precisely locating a repeatedly used insertion site within a particular gene. (2) We previously found that insertion of viral DNA into a bacterial gene is a precisely reversible process. We know this because loss (or excision) of the viral DNA lead to restoration of gene function (see last year's report). Excision from within a gene requires the same virus-specific proteins that are required for excision of λ DNA from the normal attachment site: the int and xis proteins. By increasing the power of our selective technique, we have now shown that gene function is restored in fewer than one cell in 10^{10} when int or xis protein is lacking. This result shows that excision of the viral DNA is not promoted to any measurable extent by recombination systems controlled exclusively by bacterial genes. This finding has implications with respect to the structure of the attachment site. (3) It is known that λ DNA replicating inside an infected cell exists as oligomers of several λ chromosomes joined head to tail. During morphogenesis these oligomers are cut into monomers and packaged by structural proteins of the phage. Cutting occurs at a unique site called the end-join. In circumstances where formation of oligomeric DNA is prevented, we believe that λ DNA is cut and packaged in a different way. One cut is in-

deed made at the end-join, but the second cut, instead of occurring at a second end-join, seems to occur in such a way that the amount of DNA that is finally packaged corresponds to the maximum amount of DNA that can be accommodated inside a λ head (a "headful"). We also have evidence suggesting that this method of packaging can be used, although with low efficiency, to form virus particles containing DNA derived entirely from the host chromosome. The headful mechanism of packaging is a normal feature of the life cycle of several groups of viruses not closely related to λ but had not previously been observed for λ. (4) There are at least seven phage genes (A, W, B, D, E, and F) and one host gene (groE) which are essential for the packaging of λ DNA and the formation of the λ head. We have shown that a mutation in the groE gene interferes with cell growth and drastically reduces stable RNA synthesis, in addition to its effect on phage head formation. The RNA synthesis defect, however, also requires a second as yet unidentified genetic determinant. Genetic analyses suggest that the head formation defect of groE mutants is the result of a faulty interaction between phage head proteins B and C. The faulty interaction can be partially compensated for by virus mutations which lower the level of gene E protein. Consistent with this hypothesis are the following observations: (a) the low levels of active phage made in groE hosts may lack the phage gene C protein and (b) false revertants (genetic alterations reversing the affect of a mutation but which are located at sites distinct from that mutation) of mutations in genes B and C map in gene E and visa-versa. Thus, a host function can play a role in phage λ head formation and we can begin to specify what that role is. (5) λ "head" proteins are made in vastly differing amounts, but yet there appears to be no significant difference in the mRNA levels corresponding to these proteins. This argues for some sort of translational control mechanism. We have shown that the level of a particular "head" protein (W) is controlled by the level of a second "head" protein (B). This suggests a model, one protein controlling the translation of a second functionally related protein, which could account for some of the differences in the amount of different proteins.

Significance to Biomedical Research and the Program of the Institute: The insertion and excision of viral (and other episomal) DNA is known to change the physiology of the host organism. In addition, these viruses (and other episomal elements) provide an important mechanism of genetic transfer and exchange in the microbial world. Viruses promote genetic transfer because of their ability to convert host genes to an infectious form by packaging them inside viral structural proteins. It is highly likely that such a successful symbiotic relationship has an analogy in the relationship between certain animal viruses and the cells of higher organisms. In fact it is known that animal viruses can package the DNA of their hosts although the biological significance of this phenomenon is unknown. Moreover, an understanding of the control mechanisms operative in these easily studied microorganisms has obvious relevance to an understanding of regulatory elements in higher organisms. The ability to direct the insertion of phage DNA to predetermined locations on

the host chromosome should prove to be an extremely useful and widely applicable method for isolating gene products and studying the control of bacterial gene expression.

Proposed Course of Project: We intend to further characterize the mechanism of phage DNA insertion into abnormal sites and its relation to normal insertion. We hope to construct new transducing phages for regions of the bacterial chromosome that are of particular interest (see last year's report). We plan to further characterize the phage particles which we believe are formed by the "headful" mechanism with the aim of testing the headful model for λ. We plan to continue studies initiated several years ago on the properties of a λ variant unable to circularize its DNA. We plan to determine the nature of the cellular component affected by the groE mutation and how it can affect such diverse cellular functions as stable RNA synthesis and phage λ head formation. We also intend to confirm and extend present findings of translational control for phage proteins using in vitro translation systems.

Honors and Awards: None

Publications:

1. Shimada, K., Weisberg, R. and Gottesman, M. (1973). E. coli Mutants Produced by the Insertion of Bacteriophage λ DNA. Genetics, in press.

2. Sternberg, N. (1973). A bacterial mutant defective for phage λ head formation (groE). I. Initial characterization. J. Mol. Biol., in press.

3. Sternberg, N. (1973). A bacterial mutant defective for phage λ head formation (groE). II. The propagation of phage . J. Mol. Biol., in press.

Serial No. HD-LMG-2
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Factors Influencing Genetic Transcription-Initiation and Termination

Previous Serial Number: Same

Principal Investigator: Robert J. Crouch, Ph.D.

Other Investigators: None

Cooperating Units: Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Dr. Walter Keller

Man Years

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives: The study of the coordinate expression of several genes during the development of the bacteriophages T4 and lambda has led to many current concepts of gene regulation. Several proteins have been identified as possible candidates for controlling transcription-one of these, termed "rho", acts *in vitro* by terminating transcription-thereby inhibiting the expression of genes proximal to these stopping points. Recently a second protein, kappa, has been shown to have similar properties to rho- and as reported in last years annual report, I have isolated a separate protein with similar properties but not identical to rho or kappa. The objective of the research reported in this project is to discover how these proteins are related to *in vivo* regulation-specifically by determining how the proteins function *in vitro*. A second objective reported in this project is to relate a protein, ribonuclease H (RNase H) to some cellular function. RNase H has several possible functions related to gene regulation.

Methods Employed: Rho, kappa and what I call depression factor are all purified by standard techniques of ion exchange chromatography and sizing. RNase H assays are performed by degradation of ^{32}P labeled rA·dT and separation of the degradative products by thin layer electrophoresis on polyethyleneimine sheets. This technique allow us to distinguish between RNase H and other enzymes capable of attacking the substrate.

Major Findings:

a). Progress in 1973

1. Demonstration that AMV reverse transcriptase also contains as an integral part of the protein a ribonuclease H activity. The RNase H of AMV is inhibited by antiserum against AMV reverse trancriptase but a similar RNase H from chick embryos is not inhibited by the same antiserum indicating a separate origin for the viral RNase H activity.
2. Demonstration of the presence of RNase H activity in E. coli and a search for the function of the enzyme. With the exception of one gene, all genes which now have DNA ts mutants of unknown function have been examined for altered RNase H's - with a complete negative result thus far.
3. RNA degrading activity of E. coli DNA polymerase I and T4 DNA polymerase have been studied. DNA polymerase I of E. coli can degrade RNA of DNA-RNA hybrids and also the DNA of DNA-RNA hybrids. T4 DNA polymerase is unable to degrade the RNA of DNA-RNA hybrids.
4. Ribonuclease III has been studied to separate it from RNase H of E. coli. Ribonuclease III can not degrade rA·dT. This latter finding with RNase III is either a contaminant or has some base specificity.
5. A factor from E. coli which depresses the level of RNA synthesis in vitro has been partially purified and characterized. The protein differs from rho and a similar factor, kappa, in a number of ways. Several properties indicate a function, in vitro, similar to that ascribed to rho.

b). Direction of Research in 1974

1. Continuation of E. coli RNase H studies-changes after infection with T4, T7, and λ. What happens to RNase H with ts DNA mutant shifts from permissive to nonpermissive and than back again?
2. Ribonuclease III - can it degrade DNA-RNA of ϕx and what are products of degradation?
3. Further purification of depression factor and nature of action.

Significance to Biomedical Research and the Program of the Institute: The regulation of transcription can be essential in controlling gene expression in most organisms. Certainly changes in gene activity medicated through regulation of transcription can affect normal cellular activity while failure to regulate properly or to reorient transcription can be the basis of many medical abnormalities.

Honors and Awards: (Robert Crouch)

1. Invited Lectures:
   a. Cold Spring Harbor Laboratory, July 1972
   b. University of Washington, Seattle, Feb 1973 Genetics Department
   c. Genetics "Group" Michigan State University, East Lansing Michigan March 1973

Publications:

1. Article published in Periodical
   a. Keller, W. and Crouch, R., Degradation of DNA-RNA Hybrids by Ribonuclease H and DNA Polymerases of Cellular and Viral Origin,

Proc. Nat. Acad. Sci, 69: 3360-3364, 1972

2. Article published in book
   a. Keller, W. and Crouch, R. The Relationship of Viral to Cellular Ribonuclease H, 4th Lepetit Colloq. in press.

Serial No. HD-LMG-1
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Integrative Control of Macromolecular Synthesis

Previous Serial Number: Same

Principal Investigator: Michael Cashel, M.D., Ph.D.

Other Investigators: H.A. Raue, Ph.D.
Ernest Hamel, M.D., Ph.D.

Cooperating Units: Department of Biological Sciences, Columbia University;
Department of Chemistry, University of Minnesota;
Department of Biochemistry, School of Medicine, University of Minnesota;
Roche Institute of Molecular Biology

Man Years

Total:	3.6
Professional:	2.6
Other:	1.0

Project Description

Objectives: The broad objective of this project is to understand physiological cellular response to changes in nutritional and environmental conditions. The ensueing coordinate adjustments of cellular components and activities to allow an adaptive transition to renewed cellular growth and avoidance of cellular injury. Specifically we are continuing to explore the roles played by unusual guanine nucleotides ppGpp and pppGpp which apparently can function as "coarse tuning" regulators of major cecllular synthetic processes as well as also showing some highly specific regulatory functions. As before, our approach to the role of these nucleotides is subdivided into three areas: 1) biosynthesis mechanism; 2) regulation of genetic activity and 3) regulation of metabolic activity.

Methods Employed: In addition to classical biochemical methods, solubility gradient techniques have been further refined using solid matrix immobilization

of insoluble precipitates. We are also exploring centrifugal gradient solubilization approaches which combine the solubilization gradient with a density gradient which limits diffusion of protein molecules to the point of solubilization. The permeable cell techniques discussed earlier have been optimized and now give access to new preparative routes of nucleotide synthesis as well as analytical questions. Methods for the study of the partial reactions in protein synthesis have been brought to the laboratory by Dr. Hamel. Through collaborations we have gained access to the particular expertise of other laboratories regarding carbon natural abundance nuclear magnetic resonance spectroscopy, coupled transcription-translation systems, phospholipid synthesis with insoluble membrane systems and further studies with particular homogeneous protein systems, such as EF-Tu and EF-G. Finally, knowledge of the ribosomal dependent synthesis reaction has been exploited for more efficient preparative synthesis of ppGpp as well as for synthesis of pppGpp and hydrolysis resistant derivatives, such as pcppGpp.

I. Biosynthesis of ppGpp

Early in the project year, the in vitro synthesis of ppGpp and pppGpp was reported by (Haseltine, Block, Gilbert and Webber, Harvard Univ.) to require ribosomes, GDP (or GTP) and ATP substrates, EF-G and a protein factor eluted from ribosomes with high salt. Using this starting point, we have confirmed this finding and established the identity of ppGpp produced in cells with the in vitro product. The protein factor catalyzing the ppGpp synthetic reaction has been purified to apparent homogenity as judged by SDS gel electrophoresis. The factor has a molecular weight of 80,000 in such gels and migrates as a monomer in Sephadex G-100 columns equilibrated in 1 M NH_4Cl. Under optimal assay conditions, the protein factor has a specific activity of 25 moles of product synthesized/sec/mole factor. This rate is nearly equal to the rate of peptide bond formation per ribosome. The availability of pure factor enable a determination of reaction requirements in a relatively pure system.

1. Reaction requirements have so far been determined as follows:

A) High salt washed ribosomes: A requirement for extensively (5X) washed ribosomes in a 1 M NH_4Cl is readily demonstrable. Interestingly, the ribosomal requirement for ppGpp synthesis is less fastidious than the ribosomal requirement for protein synthesis. Ribosomes can be treated so as to abolish their ability to participate in protein synthesis without altering their ability to promote ppGpp synthesis. Stoichiometric studies reveal saturation of factor activity at ribosome: factor ratios of less than one suggesting either that the factor acts catalytically or that not all the ribosomes are active. The minimum ribosomal unit capable of acting in this regard is being determined.

B) Transfer RNA and messenger RNA: With the purified system an absolute requirement for uncharged tRNA is observed and cannot be replaced by aminoacyl-

ated tRNA. This differential effect of the two forms of tRNA verifies predictions based on ppGpp synthesis in whole cells. A second RNA requirement exists which so far is not yet absolute; a requirement for messenger RNA codons. This requirement can be satisified with either polynucleotides or triplets. The codon requirement relates to the tRNA requirement in that the latter is codon-specific. Thus uncharged phe-tRNA will stimulate only in the presence of poly U but not in codons lacking UUU codons such as in $(A,G,C)_n$. The stoichiometry and structure activity relationships for the tRNA are being investigated.

C) Substrate requirements: The phosphate-accepting substrates are limited to GDP, GTP and pcppG; no other purine ribo- or deoxyribo-nucleoside 5' di- or tri-phosphate will substitute. The phosphate donating substrate seems specific to ATP and no activity is seen with dATP, ADP, XTP, ITP, pcppA or pnppA.

2. Complementation in a mutant system: Even low-salt washed ribosomes derived from mutant strains unable to synthesize ppGpp during amino acid starvation also show no synthesis of ppGpp in vitro. Addition of apparently pure factor derived from stringent strains restores the mutant capacity to enable the synthesis of ppGpp. This complementation result seemingly accounts for observations that the rel allele is dominant over the rel$^-$ allele. It seems very likely that the ppGpp synthetic factor is after all the product of the rel gene.

3. The reaction is independent of EF-G: Although the Harvard group originally reported that EF-G was required for the ppGpp synthetic reaction, there are several criteria indicating this is not the case for the purified system. The purified system is judged to not be contaminated with EF-G for lack of i) GTPase activities, ii) an appropriate band after SDS gel electrophoresis of 100 X the amounts of reaction constituents and iii) immunoprecipitation with authentic anti-G antibody under conditions which detect EF-G admixtures at ratios of 1 mole % of ribosomes. The reaction is i) not inhibited by concentrations of anti-G antibody which abolish 85% of GTPase activity of added EF-G, ii) neither stimulated nor inhibited by adding authentic EF-G to the reaction, iii) not inhibited by concentrations of fusidic acid which inhibit 90% of the GDP binding activity of EF-G and iv) not inhibited by removal of ribosomal proteins (L-7 and L-12) which are required for EF-G ribosomal dependent activity.

4. ppGpp synthesis occurs by pyrophosphorylation: Studies on the mechanism of phosphorylation of GDP and GTP to produce ppGpp and pppGpp respectively indicates that the reaction proceeds as a pyrophosphate transfer reaction rather than two successive single phosphorylations by ATP. The relative uniqueness of pyrophosphate transfers in the construction of polymeric phosphates makes this reaction of some biochemical interest, particularly because the β phosphage residue of ATP becomes inserted solely as the α residue on the ribose 3' position of the product.

5. In vitro preparative synthesis of ppGpp, pppGpp and pcppGpp: The in vitro ribosomal reaction has been exploited as a preparative tool because of its very high specific activities. In a reaction which is linear for hours, 1 mg of factor together with ribosomes catalyses the conversion of about 3-1/2 g of GTP to product every 15 minutes. The reaction allows preferential synthesis of ppGpp from GTP by inclusion of EF-G, preferential synthesis of pppGpp by inclusion of pyruvic kinase and PEP, and the synthesis of pcppGpp by substituting pcppG for GTP as the phosphate accepting substrate. The preparative route has been modified from that used with earlier fermentor cultures so as to allow reuse of the ribosomal bound factors for repeated syntheses. In this manner it is relatively simple to produce gram amounts of these nucleotides and derivatives, thus facilitating studies of their effects.

6. C13/C14 NMR structure studies: In collaboration with Drs. G. Gray and J. Bodley we have examined synthetic ppGpp under carbon natural abundance NMR spectra. Such measurements can detect the ribose positions phosphorylated as giving a broadening of a spectral peak corresponding to positions adjacent to those phosphorylated. Such spectra allow an unequivocal assignment of one pyrophosphate in ppGpp to the ribose 5' position and the other to the ribose 3' position. Thus the structure of the compound is ppG3'pp, confirming conjectures made earlier in these reports.

7. The in vitro synthetic reaction relates well to cell synthesis: The in vitro ppGpp synthetic reaction, even at this early stage of characterization apparently can account for much of what was known of ppGpp synthesis from in vivo evidence. For example, the relatively enormous specific activities are expected as are requirements for messenger RNA and uncharged tRNA. Even the existence of a pyrophosphorylation reaction was deduced from cellular chase kinetics. Of great interest is the fact that the majority of the factor is ribosomal bound to low salt washed ribosomes and not found in S 100 fractions. Also that the reaction is inhibited by tetracycline (as in cells) but not by chloramphenicol, which does inhibit ppGpp synthesis in amino acid starved cells. These last two feactures are not predicted as is the intriguing dilemma posed by the apparent specificity of the reaction for guanine nucleotides coupled with the noninvolvement of the elongation factors in protein synthesis, which are thought to generate this specificity of protein synthesis. Apparently there are alternative mechanisms of generating this specificity as well.

II. Regulation of Genetic Activity

Previous project reports have drawn parallels between the inverse relation between ppGpp levels in cells and the rates of RNA accumulation on one hand and the ability of ppGpp to specifically bind to the initiation site on RNA polymerase so as to inhibit RNA chain initiations starting with GTP on the other. We have collaborated with Dr. G. Zubay in order to explore the powerful in vitro analysis of genetic expression in crude extracts capable of coupled transcription and

translation. Such extracts are capable of hydrolyzing pppGpp to ppGpp quite rapidly, while both ppGpp and pcppGpp are very stable. The addition of ppGpp (or pppGpp which is converted to ppGpp) to this system results in promoter specific stimulation of expression of the ara, lac, and trp operons while at the same time giving inhibition of the expression of the arg operon. With the non-hydrolysable pcppGpp, no stimulations were observed, but inhibition of arg operon activity (75%) was indistinguishable from that given by ppGpp. For the lac operon no stimulation was also observed with GTP, GDP, pGp, pppGp and cGMP. These studies indicate that the stimulation of genetic expression is highly specific for the ppGpp structure, while inhibition of arg gene expression occurs with both ppGpp and pppGpp but not with GDP or GTP.

It is possible to experimentally isolate the translational component of coupled system activities by substituting purified messenger RNA in place of DNA in the cell-free system. With β-galactosidase message as well as MS 2 RNA, ppGpp and pppGpp show only a slight inhibitory effect on message-directed protein synthesis. Since these nucleotides do affect coupled transcription and translation activities of the lac operon we surmise that these effects are exerted at the level of RNA polymerase activity.

Analogous studies of the DNA-directed synthesis of $tRNA^{tyr}$ show no effect of moderate levels of ppGpp, pcppGpp or pppGpp. Even high concentrations of ppGpp, bordering on levels of any guanine nucleotide which gives non-specific inhibition, only slightly inhibits synthesis of this tRNA species. Rather surpringly it is apparent that neither ppGpp nor pppGpp act alone as negative effectors on the synthesis of this tRNA species. This is surprising since precisely this species of tRNA does have its synthesis regulated in Ø80 Su_{III}^{+} infected, amino acid starved stringent cells.

Continuing studies with essentially pure RNA polymerase interactions with nucleotides indicate that the enzyme overall has a means of minimizing binding of pppGpp to its elongation sites. If this were to occur, the compound should be an inhibitor of elongation with a potency similar to 3'deoxy ATP; however its inhibition characteristics are barely detectable on elongation. Thus there is further substantiation that both ppGpp and pppGpp act at the level of RNA chain initiation with the purified enzyme.

III. Metabolic Activity Effects

1. ppGpp binds to EF-Tu and interacts like GDP: Studies of nucleotide binding to protein synthetic elongation factor TU indicate that ppGpp can interact with this protein in essentially the same manner as GDP. Similarities in both binding and dissociation kinetics are observed. Bound GDP can exchange with ppGpp and vice versa. In the presence of EF-Ts, ppGpp bound to Tu is released and exchanged with appropriate concentrations of GTP. Again like GDP, ppGpp bound to Tu will not support the formation of a ppGpp, Tu, tRNA triplex.

2. Ability of pppGpp to substitute for pppG in protein synthesis: The extent to which pppGpp and ppGpp can substitute for GTP has been examined. ppGpp does not substitute for GTP in any reactions, but behaves analogously with GDP. pppGpp can effectively substitute for GTP in reactions catalyzed by initiation factor 2 (ribosomal binding of fmet-tRNA and formation of N-formylmethionyl-puromycin) and by elongation factor T (ribosomal binding of phe-tRNA and di-peptidyl tRNA formation). Neither pppGpp nor ppGpp was able to support poly-phenylanine synthesis. With elongation factor G, pppGpp is almost completely unable to support the formation of N-acetylphe-phe-puromycin, yet ribosomal and G dependent hydrolysis of pppGpp occurs, yielding ppGpp. This hydrolysis is mediated by G factor since it is blocked by fusidic acid as well as by anti-G antibody. The rates of EF-G dependent binding of pppGpp to ribosomes in the presence of fusidic acid was nearly the same as GTP binding. Thus the failure of pppGpp to support overall protein synthesis and polyphenylalanine synthesis can be attributed to its failure to support a late step in the translocation reaction. We are currently attempting to pinpoint this impairment.

3. Permeable cell studies: We have established that a combination of temperature shock and osmotic pressure shock renders preparations of E. coli permeable, lacking in acid extractable pools, and capable of RNA, protein and ppGpp synthesis under appropriate supplementation conditions. In such preparation, the regulatory characteristics of ppGpp synthesis mimic those of whole cells. The synthesis of ppGpp further can be closely correlated with the preservation of the capacity to synthesize proteins. The protein synthesis observed can be coupled to transcriptional events but at efficiencies about 100 times higher than with cell-free systems. In this system mRNA decay kinetics can be demonstrated to be only slightly slower than in whole cells; ppGpp synthesis is dependent upon the presence of mRNA.

The system can also be used to measure decay of ppGpp, an attribute of whole cells that so far is not obtained with in vitro systems. By allowing phosphate labeling of ppGpp, then chasing with cold phosphate the decay of radioactive ppGpp can be followed. If a complete array of amino acids is present and protein synthesis allowed by appropriate supplementation, only then does ppGpp breakdown at a rapid rate. A full complement of amino acids alone (without protein synthesis) does not lead to breakdown of ppGpp. The possible role of translocation in ppGpp decay is further suggested by the fact that fusidic acid (0.4 mM) almost completely inhibits ppGpp decay and similarly affects protein synthesis in this system. In marked contrast, S30 preparations as well as ribosomes + EF-G which are catalyzing translocation do not similarly lead to consumption of ppGpp.

Since a variety of cell evidence (summarized earlier) rather strongly suggests that ppGpp might well be a normal intermediate in protein synthesis and consumed by protein synthesis, these observations are being pursued in hopes of resolving these discrepancies between permeable cell and whole cell

behavior as compared to the inability of these nucleotides to drive protein synthesis in classical synthetic systems.

Significance of Biomedical Research and the Program of the Institute: Integrative control processes in general, and adaptive regulation to nutritional and environmental changes in particular are common to all growing cells. An understanding of these processes at any level contributes to knowledge of how cells respond to their immediate environment and its changes. Such adaptations unquestionably occur during differentiation and development and disturbance of these processes constitutes aspects of neoplastic cell transformations. The role of ppGpp has emerged as a master regulatory compound present in enormous amounts, controlled by perturbations in protein synthesis, capable of regulating genetic transcription and interacting with a variety of cellular functions that account for a sizeable part of cellular biosynthetic capacities. These include nucleotide synthesis, membrane transport, phospholipid synthesis, RNA synthesis, protein synthesis, and glycolytic fermentation of sugars.

Proposed Course of Project: We plan to pursue the mechanism of the observed specificity of the biosynthetic reaction for guanine nucleotides as well as the requirements for tRNA and mRNA codons. In addition, the ribosomal requirement for the reaction will be dissected to ascertain the minimal ribosomal components needed since ppGpp synthesis provides an alternative way of looking at ribosomal function. In addition, we shall continue our studies on the mechanism of interaction of ppGpp with gene transcription by RNA polymerase. The discrepancies revealed by comparison of *in vitro* protein synthesis with *in vivo* process will be analyzed both from the point of view of ppGpp consumption and the functional basis of the involvement of the 3' hydroxyl group of GTP in protein synthesis.

Honors and Awards:

Member American Society of Biological Chemists

Invited Speaker and Lectures

Gordon Conference on Biological Regulation Mechanisms, July, 1972
Cold Spring Harbor Symposium on Ribosomes, September, 1972
Laboratory of Biochemistry, National Heart and Lung Institute, December, 1972
Molecular Disease Branch, National Heart and Lung Institute, December, 1972
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, January, 1973
Department of Biochemistry, University of Minnesota Medical Center, Minneapolis, Minnesota, February, 1973
NICHD Institute Seminar

Publications:

1. Miller, D.L., Cashel, M. and Weissbach, H.: "The Interaction of Guanosine 5'-diphosphate,2'(3')-diphosphate with the Bacterial Elongation Factor Tu." Arch. Biochem Biophys. 154: 675-682, 1973.

2. Raue, H.A. and Cashel, M.: "Regultion of RNA Synthesis in Escherichia coli. I. Characterization of cells subjected to simultaneous temperature and osmotic shock." Accepted Biochem. Biophys. Acta.

3. Raue, H.A. and Cashel, M.: "Preparation of [^{32}P] β labeled Ribonucleoside 5' triphosphates from Permeabilized Cells." Accepted Analytical Biochemistry.

Serial No. HD-LMG-4
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: In vitro Translation of Adenovirus Messenger RNA's

Previous Serial Number: None

Principal Investigator: Heiner Westphal

Other Investigator: Lawerence Eron

Collaborating Investigators: Robert Callahan
Philip Leder

Cooperating Units: None

Man years

Total: 2.5
Professional: 1.667
Other: 0.83

Project Description:

Objectives: To study the function of genetic elements of tumor viruses in lytically infected and in transformed cells.

Methods Employed: Messenger RNA is extracted from cells containing genetic elements of the tumor virus Adenovirus Type 2, and purified by adsorption to poly-dT-cellulose. Purified RNA preparations are characterized according to size, and to content and complexity of virus genetic information. The biological activity of virus-specific messenger RNA's in directing protein synthesis in extracts derived from mammalian cells is tested in systems described elsewhere (see Dr. Leder's report).

Major Findings: The work was started in September 1972 with setting up the biochemical laboratory and the cell growth facilities. Our first experimental results were obtained a few months ago, and we can, therefore, only offer preliminary conclusions from the work done so far.
Messenger RNA extracted at late time of productive infection from KB cell cultures exposed to Adenovirus Type 2, is very active in directing protein synthesis in mammalian cell-free protein-making systems. The polypeptide synthesized in vitro display a unique and reproducible pattern of bands in acrylamide gel electrophoresis. The mRNA preparations contain about 75% of the genetic complexity of Adenovirus Type 2 DNA. Contaminating host cell mRNA's can, at least in part, be removed from the preparations by preparative

hybridization to and elution from viral DNA.

Significance to Biomedical Research and Program of the Institute: With the establishment of suitably advanced mammalian cell-free protein making systems a characterization of the functions coded for by tumor virus genetic elements is now within experimental reach. There is strong hope that an analysis of these functions will ultimately aid the understanding of the oncogenic aciton of these viruses.

Proposed Course of Project: Proteins obtained from mRNA-directed *in vitro* polypeptide synthesis will be characterized by acrylamide gel electrophoresis and by tryptic peptide analysis, using appropriate *in vivo* - virus proteins as a reference. Attempts will be made to map the structural genes coding for identifiable virus proteins. Nonstructural virus proteins may eventually be identified with the help of *in vivo* proteins, the synthesis of which is induced by virus infection.

Honors and Awards: None

Publications: None

Serial No. HD-LMG-3
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Molecular Aspects of the Differentiation of Embryonic Chick Lens Fibers in vivo and in Tissue Culture.

Previous Serial Number: Same

Principal Investigator: Joram Piatigorsky

Other Investigators: Peggy Zelenka
Leonard M. Milstone
Sonia S. Rothschild

Cooperating Units: None

Man Years

Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: The objective of this research is to describe in detail the sequence of events leading to the normal differentiation of lens fibers and to understand the regulatory mechanisms at the molecular level. The lens fiber is chosen as a model system to study cell differentiation for 2 reasons. First, it involves simple, easily assayed morphological changes (cell elongation and nuclear degeneration) and considerable synthesis of lens specific protein (80% of the cell protein is delta crystallin). Secondly, the process of lens fiber differentiation takes place in tissue culture if the medium is supplemented with serum, and is thus amenable to experimentation. We have shown in previous annual reports that lens cell elongation in culutre only occurs in epithelial cells explanted on or before the fifteenth day of development, that the elongation is inversely related to the extent of DNA synthesis and cell division, and that the early phase of cell elogation is associated with an increase in the relative proportion of delta crystallin synthesis. Evidence indicated that the higher proportion of delta crystallin synthesis is due to an increase in RNA synthesis and a greater stability of delta crystallin mRNA relative to other mRNAs. The initial doubling of cell length occurs in the absence of RNA or protein synthesis, and is related to the assembly of longitudinally oriented microtubules. Finally, we showed that the early phase of cell elongation can be stimulated by insulin in a chemically defined medium. The present report concerns our recent findings on the effect of insulin on the cultured cells, the extent of differentiation that will occur in vitro and the isolation and in vitro translation of mRNA

from embryonic lens fibers.

Method Employed: Histology was performed by conventional procedures using Carnoy's fixation and paraffin embedding; electron microscopy was done using glutaraldehyde fixation and Epon embedding. Cell length was measured from histological sections using a calibrated ocular micrometer. Delta crystallin mRNA was phenol extracted from fractionated homogenates of lens fibers of 15-day old chick embryos; it was purified by oligo-dT cellulose chromatography, analyzed by polyacrylamide gel electrophoresis and translated in a Krebs ascites cell-free system. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Major Findings: (1) The terminal phases of lens fiber differentiation can occur in tissue culture. Central regions of explanted lens epithelia from 6-day-old chick embryos were maintained in tissue culture for 4 weeks to determine the extent to which lens fiber differentiation would progress in vitro. Cellular outgrowth from the explants created 3 distinct zones; namely, a thick central zone, a thicker annular zone and a flattened peripheral zone. Cells of the central and annular zones underwent morphological and biochemical changes which correspond to the differentiation of lens fibers in vivo. The mean cell length increased a minimum of 25-fold. The nuclei in the longer cells became pycnotic; DNA remained in the nuclei but accumulated single-strand breaks. The cytoplasm became filled with a homogeneous granular matrix. Organelle density decreased, but microtubules persisted, mostly along surface membranes; free ribosomal clusters were present. There were occasional desmosomes and infoldings of cell membranes. The proportion of ribosomal RNA synthesized decreased relative to the total RNA synthesized, especially in the central zone. Finally, the proportion of delta crystallin synthesized increased to 40 to 50% of the newly synthesized protein. These data suggest that the transformation of lens epithelial cells into fibers results from a programmed differentiation which can take place in tissue culture. (2) Insulin stimulates microtubule assembly and cell elongation, but not delta crystallin synthesis. One µg/ml of insulin, like serum, stimulated a doubling of lens epithelial cell length and an assembly of longitudinally oriented microtubules; colchicine treatment inhibited this cell elongation. In contrast to serum, insulin neither promoted further lens cell elongation nor appreciably stimulated the synthesis of bulk proteins or of delta crystallin under the present conditions. These data indicate that the early morphological events of lens fiber differentiation can be initiated by insulin in a chemically defined, serum-free environment without significantly affecting protein synthesis. (3) Delta crystallin mRNA has been extracted and translated in vitro. Purified lens fibers from 15-day old chicken embryos were used as a source to extract delta crystallin mRNA, because these are relatively big (about 3 mg per fiber mass), easily dissected, and synthesize large amounts of delta crystallin; very few other proteins are made. RNA was phenol extracted from the polysomes or from the post-nuclear supernatant fraction of homogenates of the lens fibers and was subsequently eluted from oligo-dT-cellulose. The RNA which adsorbed to the column at the time application was assumed to contain stretches of poly-A at the 3'-end by analogy with similar studies of RNA extracted from other eukaryotic cells. The poly-A containing RNAs represented 3 to 5% of the RNA from polysomes and 0.3 to 0.5% of the total cellular RNA; 1 to 5 µg of poly-A containing RNA

was extracted from the cytoplasm of 100 15-day old embryonic chick lens fiber masses. The cytoplasmic poly-A containing RNAs migrated as a single band in polyacrylanide gels with a size corresponding to a molecular weight of approximately 700,000 daltons. By contrast with rRNA, the poly-A containing RNA stimulated the incorporation of radioactively labeled amino acids into protein in an ascites cell-free system; 0.5, 1 and 2 μg of the RNA stimulated proportionately greater amounts of incorporation. The incorporation had a Mg^{++} optimum of 3 mM. At least 50% of the new protein stimulated by the poly-A-containing RNA comigrated on sodium dodecyl sulfate polyacrylamide gels with delta crystallin. The other proteins whose synthesis were stimulated by the lens RNA were smaller than delta crystallin. These smaller proteins remained with the ribosomal pellet when the cell-free ascites system was centrifuged after incubation with the lens RNA and, therefore, may represent nascent delta crystallin polypeptides. Thus, mRNA can be purified from embryonic lens fibers on the basis of its poly-A content; in vitro experiments suggest that this mRNA translates mostly, perhaps entirely, delta crystallin.

Significance to Biomedical Research and the Program of the Institute: One of the difficulties of studying cellular differentiation is that embryonic systems have many unidentified variables and generally involve interaction of many cell types. The differentiation of lens fibers has the advantages that (1) it occurs synchronously in a single population of excised epithelial cells, (2) all processes examined in culture mimic those which take place in vivo during the normal development of lens fibers, (3) large amounts of easily identified lens specific proteins are involved and (4) simple morphogenetic changes can be related to biochemical events. The finding that insulin can stimulate lens cell elongation in a chemically defined medium should facilitate further analysis of the mechanism of cell elongation. These studies, then, give information on the mechanisms of cellular differentiation, which is relevant to the goals of this Institute.

Proposed Course of Project: During the next year, the action of insulin on the lens epithelial cells will be studied in detail. It is planned to purify and characterize delta crystallin mRNA and then to make a radioactive DNA sequence complementary to it to use as a probe for titrating gene dosages and quantitating delta crystallin mRNA levels in the differentiating lens fiber in vivo and in vitro. Other mRNAs will be isolated from the lens epithelial cells; these will be examined with respect to their synthesis and stability. The transport of mRNA from nucleus to cytoplasm will be followed during differentiation of the lens cells. The rates of synthesis of bulk proteins, delta crystallin and microtubule protein will be compared at different stages of differentiation. Finally, the physical and chemical properties of delta crystallin will be studied.

Honors and Awards: (Joram Piatigorsky)

1. Invited lectures:
   a. Fertilization and Gamete Physiology Training Program, Woods Hole, Mass. Aug. 1972
   b. National Eye Institute Seminar, Oct. 1972
   c. National Heart and Lung Institute Seminar, Feb. 1973.
   d. National Cancer Institute Seminar, March, 1973.

2. Co-Chairman and Speaker Microtubule Session, American Society of Cell Biology Meetings, St. Louis, Mo., Nov. 1972.
3. Invited participant and speaker, Lens Differentiation Workshop, Rochester, Michigan, March 1973.
4. Special reviewer, N.I.H. Study Section in Cell Biology, April 1973.

Publications:

1. Piatigorsky, J. and Rothschild, S.S.: Loss during development of the ability of chick embryonic lens cells to elongate in culture: Inverse relationship between cell division and elongation. Develop. Biol. 28: 382-389, 1972.

2. Piatigorsky, J., Webster, H. deF., and Wollberg, M.: Cell elongation in the cultured embryonic chick lens epithelium with and without protein synthesis: Involvement of microtubules. J. Cell Biol. 55: 82-92, 1972.

3. Piatigorsky, J.: Insulin initiation of lens fiber differentiation in culture: elongation of embryonic lens epithelial cells. Develop. Biol. 30: 214-216, 1973.

4. Piatigorsky, J., Rothschild, S.S., and Wollberg, M.: Stimulation by insulin of cell elongation and microtubule assembly in cultured embryonic chick lens epithelia. Proc Nat. Acad. Sci., USA, 70: 1195-1198, 1973.

5. Craig, S.P. and Piatigorsky, J.: Cell elongation and delta crystallin synthesis without RNA synthesis in cultured early embryonic chick lens epithelia. Biochim. Biophys. Acta, in press.

6. Piatigorsky, J., Rothschild, S.S., and Milstone, L. M.: Differentiation of lens fibers in explanted embryonic chick lens epithelia. Develop. Biol., in press.

Serial No. HD-LBC6

1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Regulated Gene Expression During Cell Growth and Differentiation (Note change in Project Title)
I. Gene expression during erythropoiesis
II. Gene expression during development of immunocompetency

Previous Serial Number: Same

Principal Investigator: P. Leder

Other Investigators:
H. Aviv
R. Callahan
J. Gielen
S. Krauss
S. Packman
J. Ross
D. Swan

Cooperating Units:
Dr. Yoji Ikawa
Viral Leukemia and Lymphoma Branch, NCI

Man Years

Total:	9
Professional:	8
Other:	1

Project Description:

Objectives: To define in genetic and molecular terms the mechanisms which regulate the flow of genetic information during cell growth and development. Specific model systems involving the differentiation of red blood cell precursors and immunocytes have been chosen for detailed studies. These serve as paradigms to view the process of differentiation as well as to investigate the organization of the chromosome and its relation to the immune process.

Methods Employed: These investigations have involved a complex, inter-disciplinary approach. The model systems that have been chosen involve the main-

tenance in culture of a unique line of erythrocytic leukemia cells, T-3-Cl2. This cell, when appropriately treated, undergoes a form of erythroid differentiation. Initial studies involving the immune system have focussed on several myeloma tumors which produce specific immunoglobulin light chains.

Our strategy has involved the isolation of specific mRNAs for globin and the immunoglobulin light chain using techniques of extraction and affinty chromatography developed in this laboratory. The purified mRNAs are converted into their DNA complements using the avian myeloblastosis reverse transcriptase, a procedure also developed in this laboratory. The DNA complements of these purified mRNAs are used in hybridization kinetic analyses to determine globin and immunoglobulin gene dosage and to quantitate respective mRNAs. Generally these procedures involve the use of tumors grown in animals, cloned lines grown in culture and the applications of sophisticated biochemical and physical chemical techniques in the study of these systems. In addition, studies have continued on the regulated expression of the bacterial protein synthetic elements. The methods involved have been described in previous progress reports.

Major Findings: (1) DNA has been synthesized which is complementary to mouse, rabbit, human and duck globin mRNA. This material exhibits the specific hybridization properties expected of a true copy of the respective globin genes. (2) Using these radioactive cDNAs as probes, the evolutionary relationship among these organisms has been determined at the level of the globin genes. (3) Again using the cDNA probes, the number of genes corresponding to globin in duck and mammalian cells has been determined to be no more than 3 to 5 globin gene copies per diploid genome. Thus, the amplification hypothesis of differentiation has been ruled out. (4) Using an *in vitro* culture system, the rate-limiting events in the expression of the globin genes has been identified. Globin mRNA is absent in cells prior to the induction of differentiation. After induction, 3,000 to 6,000 molecules of globin mRNA can be detected in cell culture systems. (5) Further progress has been made in the purification of the immunoglobulin light chain mRNA. (6) Immunoglobulin light chain mRNA has been translated in a cell-free system. (7) A precursor of light chain, comprising 10 to 15 more amino acids, has been identified in the cell-free system. (8) The immunoglobulin light chain message has been shown to contain a poly(A) sequence and has been shown to direct the synthesis of both constant and variable regions of the light chain. A post-transcriptional joining of cariable and constant regions has therefore been ruled out. The message has been shown to be monocistronic. (9) DNA complementary to the myeloma mRNA has been synthesized and characterized. (10) The complementary DNA has been used to assess the purity of mRNA preparations. The mRNA has been found to be less than 10% pure. (11) Preparative hybridization has been used to prepare a purified light chain cDNA. This material has been used in hybridization analyses of the constant region genome in myeloma tumors. These studies, though in progress, suggest the presence of a highly reiterated sequence. (12) The

cell-free protein synthesizing system has been used to translate bacteriophage mRNAs, laying the groundwork for a test system for identifying suppressor mutants in mammalian cells. (13) The cell-free system has also been used in order to translate mRNA derived from adenovirus infected cells.

Significance to Biomedical Research and the Program of the Institute: These studies are directed towards understanding the fundamental processes involved in the expression of genetic information. These processes is obviously relevant to the normal and abnormal embryonic differentiation, maturation and to the orderly development and function of the human organism. In addition to the relevance of these studies to inherited disorders, the work is also relevant to the process of oncogenesis as well as to disease processes which are polygenic such as diabetes, hypertension and susceptibility to oncogenic disorders. Our current findings have provided a means for studying the regulated expression of specific genes in higher organisms. These are particularly relevant to determining the pathophysiology of thalassemia. These techniques may also prove useful in the direct transmission of genetic characteristics from cell to cell in vitro

Proposed Course of Project: Having identified the transcriptional reaction as essential to the regulated expression of globin genes, this process will be studied in detail. We hope to establish a cell-free system for the transcription of globin genes directly from chromatin. These will be used to determine and identify specific regulatory elements involved in the expression of these genes. Further studies will be directed toward identifying a coordinated set of genes, regulated together with globin. Specifically these will involve enzymes required for the biosynthesis of heme. We also hope to isolate or transform mutant cell lines affected in their ability to undergo erythrodifferentiation.

Further studies will be required in order to purify completely immunoglobulin mRNA. A variety of techniques involving the isolation of nucleic acids will be employed. When immunoglobulin light chain mRNA of sufficient purity is obtained, it will be transcribed into cDNA and studies will be undertaken to account for the constant and variable regions of immunoglobulin molecules in genetic terms. Work will also proceed directed toward identifying the products of adenovirus mRNAs, in collaboration with the laboratory of Dr. Heiner Westphal. These will generally involve the characterization of tryptic peptides derived from cell-free systems, and their comparison with authentic viral proteins.

Honors and Awards: Invited Lectures

Gordon Conference on Animal Cells and Viruses, August, 1972
Department of Oncology, Columbia University, September, 1972
Plenary Lecturer, German Biochemical Society, October, 1972
Department of Biochemistry, Brandeis University, November, 1972

Department of Biochemistry, Yale University, November, 1972
Department of Embryology, Carneige Institution of Washington, Baltimore, Maryland, December, 1972
Department of Cellular Biology, Albert Einstein College of Medicine, December, 1972
Symposium Lecturer, American Association for the Advancement of Sciences, December, 1972
Department of Biochemistry, Harvard Medical School, February, 1973
Rockefeller University, February, 1973
Department of Biochemistry, Princeton University, March, 1973
Invited Lecture, Current Topics in Biochemistry Lecture Series, National Institutes of Health, March, 1973
Invited Participant, Fogarty Center Conference on Cultured Cells, April, 1973
Invited Seminar Lecture, American Society of Immunologists, Atlantic City, April, 1973
Department of Microbiology, Medical School, Washington University, St. Louis, May, 1973
President, Foundation for Advanced Edcuation in the Sciences, Inc., Bethesda, Maryland
Member, Study Section on Physiological Chemistry, National Institutes of Health, Bethesda, Maryland
Invited Participant, Work Shop on Protein Biosynthesis, Aarhus, Denmark, June, 1973
Chairman-Elect, Gordon Conference on Nucleic Acids, 1974
Invited Lecturer, Cold Spring Harbor Symposium on Chromosome Organization, June, 1973

Publications:

1. Callahan, R. and Leder, P.: In Vitro Initiation of Coliphage T7mRNA. Arch. of Biochem. & Biophys., 153: 802-813, 1972.

2. Leder, P., Skogerson, L.S. and Callahan, R.: Translation Initiation: Defects Arising in E. coli Infected with Phage T7, λ and Qβ. Arch. of Biochem. & Biophys. 153: 814-822, 1972.

3. Swan, D., Aviv, H. and Leder, P.: Purification and Properties of Biologically Active mRNA for a Myeloma Light Chain. Proc. Nat. Acad. Sci. U.S.A. 69: 1967-1971, 1972.

4. Boime, I. and Leder, P. Protein Synthesis Directed by Encephalomyocarditis Virus mRNA. III. Discrete Polypeptides Arising from a Monocistronic Messenger In Vitro. Arch. of Biochem. & Biophys. 153: 706-713, 1972.

5. Aviv, H., Boime, I., Loyd, B. and Leder, P.: Translation of Bacteriophage Qβ mRNA in a Murine Krebs II Ascites Tumor Cell-Free System.

Science 178: 1293-1295, 1972.

6. Aviv, H., Packman, S., Swan, D., Ross, J. and Leder, P.: In Vitro Synthesis of DNA Complementary to mRNA Derived from a Light Chain-Producing Myeloma Tumor. Nature 241: 174-176, 1973.

7. Packman, S., Aviv, H., Ross, J. and Leder, P.: A Comparison of Globin Genes in Duck Reticulocytes and Liver Cells. Biochem. Biophys. Res. Commun. 49: 813-819, 1972.

8. Ross, J., Ikawa, Y. and Leder, P.: Globin mRNA Induction During Erythroid Differentiation of Cultured Leukemia Cells. Proc. Nat. Acad. Sci., U.S.A. 69: 3620-3623, 1972.

Serial No. HD-LB20
1. Laboratory of Molecular Genetics
2.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Regulation of Antibody Biosynthesis
I. Mechanisms of consignment of cells to determinant-specific immunocompetency
II. Promoters and amplifers of antibody biosynthesis in cell culture
III. Initiation of determinant specific antibody biosynthesis in cell culture
IV. Deviation of immunocompetent cells

Previous Serial Number: Same

Principal Investigator: E.E. Hanna

Other Investigators: C. Lambert

Cooperating Units: None

Man Years

Total:	24/12
Professional:	24/12
Other:	0

Project Description

Objectives: To clarify postulated pathways of antibody biosynthesis and to explore alternative pathways to cellular biosynthesis and secretion of antigen induced antibodies. This involves delineating the roles of non-antibody-secreting cells, such as the thymus influenced or derived lymphoid cell (T-cell) and the more remote non-lymphoid cell type, the macrophage. The latter is a cell known to make contact with antigen first during natural or artifical immunization. We hope to learn more about the nature of the active principle in homologous serum which seems to be a required supplement in rabbit lymphoid cell cultures. If small amounts of Ig or subunits of Ig are required to initiate antibody biosynthesis in culture and the synthesized product can be shown to be distinct from these, we will have essentially defined a new Ig primer. It will then be of interest to understand its mechanism of action and its origin. [Eg., it would be interesting to compare such an Ig to receptors at the surface of Ig-secreting cells (B-cells) and to the very rarely demonstrated receptor at the surface of T-cells]. It is possible that such a receptor Ig has its activity associated in some manner with the macrophage cell type.

We wish to pursue further the role and the origin of certain cyclic nucleotides found to stimulate or amplify B-cells. We wish to determine relative

efficacies of cAMP and cGMP to amplify B-cells (proliferation and secretion). This is important because of a very current debate over which of the two compounds is the better modulator of lymphocyte activity.

As solutions to the problem of how cells initiate antigen specific Ig secretion, we will turn our attention toward developing more direct means of selectively regulating the antibody mechanism at the cellular level.

Methods Employed: Antibody secreting cells (B-cells) are detected as discrete holes (plaques), in a confluent lawn of erythrocytes bearing native and chemically linked haptenic determinants. B-cell precursors, with receptors at their surface, may be detected by the rosette test and other similar adherance tests. Detection of T-cells remains a problem since their presence is determined indirectly as a synergistic activity. Manipulation of T-cells is presumed when one has enhanced or suppressed this activity. We have prepared anti-theta antisera using both neonatal and adult thymus cells as antigen. We hope to obtain a more efficient T-cell inactivator by selective absorption and by pooling these antisera. Since concanavalin-A (Con-A) binds preferentially to T-cells, we have prepared very potent anti-Con-A antisera which in concert with a fluorescent label may be helpful in identifying T-cell populations. Experiments will continue to be preformed both *in vivo* and in cell culture.

Density gradients and antigen-specific, affinity columns are still the best available methods for isolating and harvesting cell populations, but they are by no means fully satisfactory. We will continue to seek better isolation and cloning methods.

The replica-hapten-specific plaquing method developed by us is still the method of choice for assessing B-cell secretions with respect to their specificities and also for monitoring any potential cloning method. Agar-gel and immunoelectrophoretic procedures will be employed as needed.

The ability to recover immunocompetent cells from liquid nitrogen storage with essentially the activity of fresh material greatly facilitates our studies since it allows us to repeat experiments on the same biological material.

Major Findings: We have demonstrated that dibutyryl-cAMP can amplify the number of B-cells responding to immunization (in press). In these studies a T-cell dependent antigen was used in both thymectomized (Tx) mice and sham Tx mice. The result was enhancement of the number of secreting B-cells in each group. Because of this it is important to ascertain whether cyclic nucleotides are mediators of T-cell function or whether they are associated in another fashion.

We have interpreted some of our earlier findings from studies of immunosuppression by a streptococcal toxin as a possible selective effect upon suppressor T-cells. B-cells are presumed to recover from the cytotoxin earlier than suppressor T-cell. This temporary lack of suppressor cells would allow for the observed enhancement. We will test this alternative using T-cell independent antigens, such as the purified pneumococcal capsular antigen in Tx animals and in congenically athymic animals.

We have found an important artifact in the rabbit spleen cell culture

procedure used to study antibody biosynthesis. Homologous rabbit serum is a reported nutritional requirement in such cultures. Yet, we found that all sera effective in this respect contained pre-formed, "background" anti-sheep erythrocyte (SE) antibody. This antibody was shown to sensitize SE added as antigen to immunize cultures. The sensitized SE form micro-aggregates which are lysed by complement in the assay procedure. This forms well-defined plaques which are artifacts and are eronously enumerated as antibody forming cells (Lambert and Hanna-manuscript in preparation). This artifact is probably present in the data of published reports. Publication of this result should stimulate reassessments of other reports on antibody biosynthesis which were quantitated in culture by a plaque procedure. Our future direction will be to determine if a small amount of transferred antibody (or subunits) may serve an initiator or primer functions as discussed above.

We have found that the antibody required to develop and visualize 7S IgG secreting PFC populations is itself a high affinity IgG with major anti-Fc activity. The active component of an antiserum with strong suppressive activity for 19S IgM PFC Is a high affinity IgG with major anti-Fab activity. The amount of anti-light chain activity within the anti-Fab antibody is important in this respect (manuscript in preparation). In preparations to be made in the future, these target subunits of Ig will be isolated and used as antigen to produce the particular anti-Ig reagent desired. These findings may have relevance also in initiation or suppression of certain Ig responses.

Results of our study which suggested combining combining site overlap of IgG into adjacent native determinants of the carrier moiety of a hapten-carrier immunogen were equivocal. We decided therefore to repeat the experiment with a more comprehensive immunization schedule, involving injections of immunogens consisting of reciprocal non-cross-reacting carriers. These procedures have been carried out, the cells have been harvested, and cryogenically stored. Assay of these cells should confirm or negate our original conclusions.

Significance to Biomedical Research and the Program of the Institute: Ability to regulate or modulate specific antibody responses will have great advantage over current measures of generalized immunosuppression methods in current use. It should be possible to selectively suppress a response, e.g., to a grafted tissue or organ, while maintaining desirable immunologic surveillance against disease producing immunogens. Hopefully it should be possible to enhance this surveillance. Our findings should also complement and clarify scientific thought on cellular control mechanisms in general.

Proposed Course of Project: I have chosen to point out the future direction of each facet as it was described for sake of clarity and conciseness.

Honors and Awards

Invited to present a research lecture at Howard University
Elected to membership in the American Association of Immunologist (AAI).
Represented the American Association of Immunologists in the ad hoc committee meeting with Black Scientist, sponsored by the Federation of American Societies for Experimental Biology (FASEB).
August 1972, selected by the National Research Council to give advisory

service representing scientist and engineers from minority backgrounds.

Publications:

1. Uzunoya, Anelia, D., and Hanna, Edgar, E.: B-cell Amplification by N^6, O^2 -dibutyrl Adenosine-3'-5' Cyclic Posphate in Mice. Cellular Immunology, in press.

2. Hanna, Edgar, and Watson, Dennis W.: Enhanced Immune Response Following Immunosuppression by Streptococcal Pyrogenic Exotoxin. Infection and Immunity, in press.

NICHD Annual Report
July 1, 1972 through June 30, 1973
Laboratory of Biomedical Sciences

The Laboratory of Biomedical Sciences conducts basic research in developmental enzymology, intermediary metabolism, physiological controls, and developmental pharmacology. The objective of these investigations is the elucidation of the molecular basis for selected types of mental and physical developmental pathology.

In the Section on Developmental Enzymology interest centers on the separation, purification, and characterization of enzymes from the human placenta and from fetal tissues in both normal and abnormal pregnancies. A study of the properties of pregnancy-associated enzymes from normal and abnormal gestations may give information about the etiology of reproductive disorders such as spontaneous or habitual abortions, stillbirths, or molar pregnancies.

Optimal assay conditions were ascertained and specific activities determined for the following placental enzymes: alkaline phosphatase, lactate dehydrogenase, hexokinase, phosphoglucomutase, phosphoglucose isomerase, catechole-O-methyltransferase, and two enzymes of the degradative pathway of lysine (saccharopine dehydrogenase and saccharopine reductase). Zymogram techniques were devised for detecting isoenzyme variation among the first five enzymes. These enzymes were qualitatively and quantitatively compared in normal term placenta and malignant trophoblast in tissue culture. Two striking differences were noted: the tissue in culture is virtually devoid of heat stable (placental) alkaline phosphatase, and it has an unusual lactate dehydrogenase isoenzyme. Studies designed to characterize these enzyme differences are progressing.

Investigators in the Section on Intermediary Metabolism develop laboratory analogs of mental retardation through the use of specific chemical agents and enzyme inhibitors, investigate the biosynthesis and degradation of macromolecules in neural tissue, elucidate mechanisms of transport of small molecules into and out of the brain, and evaluate the effects of excesses or deficiencies of intermediary metabolites on cellular differentiation and organogenesis (particularly of the brain). The studies in the Section range from classical biochemical investigations of enzymes of neurochemical interest and pharmacological efforts to inhibit or stimulate these enzymes to behavioral experiments on animals in which the activity of such enzymes has been altered or inhibited. In this way we hope to provide information on the basic biochemistry of the brain as well as on the conditions which alter the normal brain functions such as various genetically-transmitted mental retardation syndromes.

Among recent advances made by the Section are: (1) the further elucidation of the biochemical, pathological, developmental, and behavioral aspects of the rat model of phenylketonuria; (2) the purification and characterization of bacterial tryptophan hydroxylase and bacterial dihydropteridine reductase, two enzymes involved in bacterial hydroxylation; (3) the preparation and characterization of poly-A containing RNA's from rat brain and a study of

their function in information transfer vis-a-vis brain messenger RNA; (4) The demonstration and characterization of a new protein factor involved in stimulating pteridine biosynthesis in bacterial systems; (5) The development of new methodology for the study of purine phosphoribosyltransferase, the enzyme involved in the Lesch-Nyhan syndrome, and the study of this enzyme in rat brain.

The Section on Physiological Controls studies basic endocrine and neuroendocrine mechanisms and factors controlling their development. A focus on synthesis and mode of action of cyclic AMP reflects the potential importance of this ubiquitous intracellular regulator to our understanding of metabolic regulation, cell growth, and differentiation. Most studies utilize two metabolic pathways as models for delineating interactions between hormones, neurotransmitters, and nutrients. Protein phosphorylations controlling glycogen metabolism provides us with a well-characterized model of cyclic AMP action; indole metabolism in the pineal gland has proved excellent for studying the mode of action and synthesis of neurotransmitters.

During the past year, we have obtained partial or complete purification of all of the enzymes known to participate in glycogen cycle phosphorylations. Of major significance was the observation that glycogen synthetase phosphatase is a general phosphoprotein phosphatase that dephosphorylates phosphorylase-b kinase and other protein substrates of the cyclic AMP-dependent protein kinase. The development of fetal rat liver glycogen synthesis has been reproduced in a completely defined organ culture system and shown to be related to the development of glycogen synthetase. In these studies, separate and sequential roles for hydrocortisone and insulin have been characterized.

A new method for measuring tryptophan hydroxylation was developed and used to show that this rate-limiting enzyme in serotonin synthesis in the pineal gland is controlled by circulating tyrptophan levels. Norepinephrine was shown to decrease pineal serotonin levels by a sequence of reactions involving a specific receptor, cyclic AMP, and the induction of serotonin N acetyltransferase. Antigonadotrophic affects of the pineal gland have been further delineated. A potent antigonadotrophic compound produced by the pineal gland was purified and shown to be a small peptide.

The Section on Developmental Pharmacology studies the subcellular processes involved in the "induction" of pharmacologically important enzymes by drugs, polycyclic hydrocarbons, or insecticides (i.e., xenobiotics). The combination of these environmental stimuli and the host's genetic constitution therefore influence the levels of these mono-oxygenase activities in maternal tissues, in the placenta, and transplacentally in fetal tissues. Consequently, we are concerned with (1) the interplay between genetic expression and environmental stimuli, (2) possible effects of xenobiotics on normal fetal growth and development, (3) mechanisms of "pharmacological immaturity" of the premature and newborn, (4) mechanisms of disease of the fetus and newborn involving oxidative and conjugative metabolism of normal body substrates such as steroids and bilirubin, and (5) possible therapeutic and diagnostic procedures for aiding and predicting pharmacogenetic disorders and teratogenic effects in the fetus and neonate.

With fetal cell cultures derived from the entire hamster or mouse, from rat or mouse liver, and most recently from established cell lines of rat or mouse hepatoma or of normal liver origin, we have developed an experimental model having numerous advantages over studies in the intact animal. We have determined that the "induction," or stimulation, of mono-oxygenase activity such as aryl hydrocarbon hydroxylase and other drug-metabolizing enzymes by phenobarbital, polycyclic hydrocarbons, or biogenic amines is controlled at two levels: the level of transcription involving gene activation, and a posttranscriptional effect in which the normal rate of decay of induced enzyme activity is retarded. The induction process effected by phenobarbital is more dependent on ribosomal RNA synthesis than that effected by either aromatic hydrocarbons or biogenic amines.

With the use of inbred strains of mice, we have determined that the "induction" of at least 7 drug-metabolizing enzyme activities can segregate as a single autosomal dominant trait, can be dominantly repressed, or can be expressed as a codominant trait, depending on the inbred strains crossed. A new hemoprotein, cytochrome P-448, is concomitantly synthesized during the induction process. In mice in which the induction process and P-448 formation are relatively "nonresponsive" to aromatic hydrocarbons such as 3-methylcholanthrene or 5,6-benzoflavone, these parameters are fully expressed by administration of a compound having extremely high affinity for all tissues, 2,3,6,7-tetrachlorodibenzo-p-dioxin; it thus appears that the Ah locus in "nonresponsive" mice can respond normally perhaps because the deficient "receptor site" is made to function by an inducing compound of sufficiently high affinity. The relative presence of "aromatic hydrocarbon responsiveness" in certain mice results in more 3-methylcholanthrene-initiated tumors, in a shortened sleeping time when given certain drugs, in a shortened survival time when dosed with large amounts of polycyclic hydrocarbons, and in an increased protection against the toxicity of certain insecticides. These genetically mediated dissimilarities in xenobiotic metabolism may be a useful probe in demonstrating differences in the rates of formation of certain drug-produced birth defects.

Serial No. HD-LB1(c)

1. Laboratory of Biomedical Sciences
2. Section on Developmental Enzymology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Developmental Enzymology

Previous Serial Number: Same

Principal Investigator: J. C. Robinson

Other Investigators: C. Chen
J. B. Edlow
T. Fjellstedt
T. Ohta
J. R. Relacion

Cooperating Units: P. O. Kohler, Reproduction Research Branch, NICHD

Man Years

Total:	48/12
Professional:	48/12
Other:	0/12

Project Description:

Objectives: The main objectives of this project are: (1) to study qualitative and quantitative changes in selected enzyme systems in the developing placenta and fetal organs in normal gestation; (2) to study qualitative and quantitative changes in these same enzyme systems in the developing placenta and fetal organs in reproductive disorders such as abortions, stillbirths, and fetal malformations; (3) to isolate. purify, and characterize these enzymes from the normal placenta; and (4) to identify and characterize pregnancy-associated enzymes in maternal blood plasma.

Methods Employed: The principal methods employed are: (1) the zymogram technique, by which electrophoretic variants of enzymes can be detected; (2) autoradiography, with which potential carrier function is evaluated (for example, by using I^{131}-tagged thryroxine); (3) enzyme assays, which are usually based on spectrophotometric or radiometric methods; (4) protein isolation techniques, including salt- and solvent-fractionation, gel-filtration, and ion-exchange chromotography; (5) paper and thin layer chromatography; (6) tissue culture; and (7) immunochemical procedures.

Major Findings: (1) The specific activities and electrophoretic patterns of three carbohydrate-metabolizing enzymes--hexokinase, phosphoglucomutase,

and phosphoglucose isomerase--are essentially the same in cultured choriocarcinoma cells as in term placenta. (2) In addition to the 5 lactate dehydrogenase (LDH) isoenzymes characteristic of term placenta, the choriocarcinoma zymogram has an additional substrate-dependent band between LDH-2 and LDH-3. (3) Heat-stable alkaline phosphatase is strikingly reduced in cultured choriocarcinoma cells as compared with term placenta. This low basal activity was increased approximately 20-fold by the inclusion of 5-bromodeoxyuridine in the culture medium. (4) We demonstrated that the enzymes involved in the initial steps of lysine metabolism are present in human placental tissue. Optimum stability and assay conditions were established for the enzymes involved in the first two sequential reactions (lysine to saccharopine and saccharopine to α amino adipic-δ-semialdehyde), and Michaelis constants were determined for the substrates used in the spectrophotometric assay. Reaction products were confirmed by formation of chemical derivatives, where appropriate, and by thin-layer chromatography. Initial purification procedures of ammonium sulfate fractionation and DEAE-cellulose column chromatography resulted in approximately a 100-fold purification of these enzymes. (5) A quantity of crystalline saccaropine sufficient for a wide range of experiments was prepared biosynthetically and purified by ion exchange chromatography, paper chromatography, and repeated crystallization. (6) No phenylethanolomine-N-methyl-transferase activity was found either in human term placenta or rat term placenta. (7) The mean specific activity (n mole metanephrine formed/hr/mg wet weight) of catechol-O-methyl transferase (COMT) of seven human term placentas was 0.58. The range was 0.46 to 0.68. (8) In a preliminary study, COMT specific activity in rat placenta decreased significantly from 2.91 on gestational day 12 to 1.88 on day 14. The specific activity was still lower (1.62) on day 16. A slight rebound was noted from day 18 (1.91) through day 20 (2.11).

Significance to Biomedical Research and the Program of the Institute: The study of placental enzymes in normal pregnancy will provide a suitable baseline with which one can compare the same enzyme systems in placental explants, in benign and malignant trophoblast cells in culture, and in placental tissue from reproductive disorders such as stillbirths and abortions. For example, consistent changes in enzymes in abortion material may reflect differences in placental function, which in turn could affect fetal viability. Hopefully, these studies will lead to increased understanding of both normal and abnormal biochemical processes involved in pregnancy and fetal development.

Proposed Course of Project: (1) Characterization of selected enzymes from placenta, maternal plasma, amniotic fluid, and trophoblast cells in culture will be undertaken. (2) Correlations between abnormal pregnancies and variations in fetal or maternal enzyme systems will be sought.

Honors and Awards: None

Publications:

1. Edlow, J.B., Kohler, P.O. and Robinson, J.C.: Choriocarcinoma *in vitro*: Evidence for exchange of enzymes between culture medium and cells. Proc. Soc. Exp. Biol. Med. 141: 798-801, 1972.

Serial No. HD-LB9

1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Models of Mental Retardation

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators: A. Andersen
V. Rowe

Cooperating Units: S. Zigler, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina
J. Sidbury, Department of Pediatrics, Duke University Medical Center, Durham, North Carolina

Man Years

Total:	27/12
Professional:	27/12
Other:	0/12

Objectives: The objectives of this research are: (1) to produce biochemical models of mental retardation syndromes in laboratory animals by chemical means; (2) to investigate the pathological and behavioral aspects of the chemical treatments; (3) to study the mechanisms by which specific chemical alterations produce a change in normal neural functioning; (4) to attempt new methods for preventing such alterations hopefully pointing the way for new clinical approaches.

Methods Employed: The methods employed include standard enzymatic assay techniques, histological examinations, and behavioral evaluation.

Major Findings: (1) Treatment of newborn rats with p-chlorophenylalanine plus phenylalanine produces a biochemical picture similar to that seen in clinical phenylketonuria, e.g. high blood phenylalanine-to-tyrosine ratio, phenylketones in the urine, low phenylalanine hydroxylase levels, lowered brain serotonin. (2) Such treatment during the development of the rat (0-21 days) produces, in the adult animal, behavioral and pathological alterations analogous to clinical phenylketonuria, e.g. hyperactivity, deficiencies in problem solving, smaller brains. (3) Such treatment also produces a delay in developmental milestones, e.g., eye opening, histological changes in the brain, and heightened cerebral excitability in the infant, treated rat. (4) The treated rat metabolizes the phenylalanine load to abnormal products characteristic of the clinical state; in the 21-day old rat the major

metabolite has been shown to be phenyllactic acid. (5) Cataracts appear in the adult rats several months after termination of the treatment; the pattern of free amino acids and of soluble proteins in the lenses of these cataractous animals is altered in a characteristic and reproducible way.

Significance to Biomedical Research and the Program of the Institute: No adequate model of mental retardation has yet been produced in a laboratory animal. The need for such a disease analog is great, since only then can investigations be initiated toward understanding the biochemical basis for the retardation. The endeavor to produce a chemical model in this Laboratory centers around phenylketonuria because it is the best characterized from a biochemical and a behavioral aspect, and because a great deal is known about the enzyme which is deleted, phenylalanine hydroxylase. If such a model can be produced, substantial information should be available about the causes and perhaps the prevention of the retardation.

Proposed Course of Project: (1) Attempts to exacerbate the lesion by prenatal administration of the p-chlorophenylalanine and phenylalanine. (2) Attempts to characterize chemically the metabolic lesion by administering one of the products of the abberant metabolism, e.g., phenyllactic acid, to see if it causes the same behavioral alterations in the adult rats. (3) Attempts to prevent the defect from developing by simultaneously administering other materials, e.g., 5-hydroxytryptophan. (4) Attempts to alleviate at least the secondary effects of the disease, e.g., skin rash and irritability, in adult phenylketonuria by altering the amino acid balance of the blood. (5) Attempts to produce other behavioral anomalies in rats, e.g., the Lesch-Nyhan Syndrome, by a similar approach.

Honors and Awards: None

Publications:

1. Andersen, A. and Guroff, G.: Enduring behavioral changes in rats with experimental phenylketonuria. Proc. Nat. Acad. Sci., U.S.A., 69: 863-867, 1972.

2. Andersen, A., Abramowitz, A., and Guroff, G.: Biochemical and behavioral changes in rats with experimental phenylketonuria. In Barchas, J. (Ed.): Behavioral Effects of Changes in Level of Brain Serotonin. Palo Alto, Academic Press, in press.

3. Andersen, A. and Guroff, G.: An animal model of phenylketonuria. Am. J. Pathol., in press.

Serial No. HD-LB10

1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mechanism of Aromatic Hydroxylation

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators: C. Letendre
C. Williams
G. Dickens

Cooperating Units: None

Man Years:

Total:	39/12
Professional:	27/12
Others:	12/12

Project Description:

Objectives: The objectives of this research are: (1) to provide information on the process of enzymatic hydroxylation; and (2) to get detailed information on the nature of phenylalanine hydroxylase.

Methods Employed: The methods employed include standard techniques of enzyme kinetics and purification, antibody detection, and organic synthesis and characterization.

Major Findings: (1) At least four different bacterial species produce an inducible phenylalanine hydroxylase; (2) Chromobacter will also produce a tryptophan hydroxylase which is not the same enzyme as its phenylalanine hydroxylase: (3) Chromobacter tryptophan hydroxylase is a pteridine-requiring enzyme similar to the one found in mammals for the synthesis of serotonin; it had never been obtained from bacteria before; (4) dihydropteridine reductase, an enzyme functioning in the coupled phenylalanine hydroxylase system, is also inducible by phenylalanine in Comamonas; it has been purified and characterized from this organism.

Significance to Biomedical Research and the Program of the Institute: Information about the phenylalanine and tryptophan hydroxylases and about ways to inhibit or stimulate them relates directly to the general problems of normal neural function and of mental retardation. The phenylalanine

hydroxylase is the missing enzyme in the best characterized of the genetic retardations, phenylketonuria. The tryptophan hydroxylase is the first and rate-limiting enzyme in the biosynthesis of the neurohumoral agent, serotonin. Detailed analysis on the structure and mechanism of these enzymes will provide new and unique information since relatively little has been accomplished on hydroxylases in general and few, if any, pteridine enzymes have been reported as homogeneous preparations.

Proposed Course of Project: (1) Continued study of the purification, mode of action, and structure of the bacterial enzymes phenylalanine hydroxylase, tryptophan hydroxylase, and dihydropteridine reductase.

Honors and Awards: None

Publications: None

Serial No. HD-LB11

1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biosynthesis and Function of Pteridines

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators: J. Cone
J. Plowman

Cooperating Units: None

Man Years:

Total:	27/12
Professional:	15/12
Other:	12/12

Project Description:

Objectives: The objectives of this research are: (1) to provide information on the enzymes in the biosynthetic pathway to the pteridine ring; (2) to investigate the functions of pteridines in addition to their action as hydroxylase cofactors.

Methods Employed: The methods used include standard techniques of enzyme purification, isotope measurement, high-voltage electrophoresis, and fluorescence detection.

Major Findings: (1) The first enzyme in the pteridine biosynthetic pathway of Comamonas has been fractionated into two protein portions; one seems to have a stimulating effect on the other and may be involved in altering the stereochemistry of the product produced.

Significance to Biomedical Research and the Program of the Institute: The pteridine cofactors are important in a number of hydroxylase reactions including those leading to the neurohormones serotonin and norepinephrine. Information about the pharmacology and biochemistry of these biosynthetic enzymes may lead to the ability to inhibit or stimulate these pathways and perhaps to new therapeutic tools for maladies involving these neurohormones.

Proposed Course of Project: (1) Purification of the two portions of the first enzyme and elucidation of the function of each; (2) purification and characterization of other enzymes of pteridine metabolism; (3) attempts to link

pteridines or pteridine derivatives to the enzymes they serve; (4) study of pteridine biosynthetic enzymes in photosynthetic organisms; (5) search for other functions of pteridines.

Honors and Awards: None

Publications: None

Serial No. HD-LB12

1. Laboratory of Biomedical Sciences
2. Section on Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Biochemistry of Brain Macromolecules

Previous Serial Number: Same

Principal Investigator: G. Guroff

Other Investigators: J. DeLarco
A. Abramowitz
S. Nakagawa
K. Bromwell

Cooperating Units: None

Man Years:

Total:	51/12
Professional:	39/12
Others:	12/12

Project Description:

Objectives: The objectives of this research are: (1) to investigate the biochemistry of brain macromolecules; (2) to determine if any aspects exist that are unique to brain; and (3) to find out whether any unique aspects are related to the unique function of the brain.

Methods Employed: The methods involved in this study include isotope techniques, high voltage electrophoresis, and tissue slice metabolic studies, as well as standard methods for purification and investigation of macromolecules.

Major Findings: (1) Brain "messenger" RNA's have been purified by their ability to bind to oligo(dT)-cellulose; (2) the RNA binding properties of various celluloses have been described and explained; (3) brain "messenger" RNA has been described in terms of its size, its poly(A) sequences, and its ability to be translated by a protein synthesis system; (4) synaptosomal RNA has been purified and its properties studied; its resemblance to mitochondrial RNA has been noted; (5) differences between the messenger fraction from the brains of immature and of adult animals with respect to the length of the poly(A) sequences have been found.

Significance to Biomedical Research and the Program of the Institute: Substantial evidence exists that brain macromolecules are involved in the

unique functions of the brain. A continuing question exists as to the directness of this involvement. These studies may shed light on this question by finding properties of brain macromolecules compatible with a unique function such as the storage of memory. Further, development of such systems as the tissue slice for RNA synthesis may provide tools for the investigation of the biochemical alterations occurring in certain types of mental retardation.

Proposed Course of Project: (1) Further characterization of rat brain messenger RNA; (2) attempts to understand the differences in the messenger fraction from young and from adult rats; (3) attempts to isolate messenger RNA's for brain-specific proteins; (4) separation of the various cell types from brain, and study of their messenger RNA's.

Honors and Awards: None

Publications:

1. Guroff, G.: Biochemistry of the brain. In Mason, H.S. (Ed.): Functional Biochemistry. MacMillan Co., in press.

2. Guroff, G.: Special aspects of amino acid metabolism. In Albers, R.W., Siegel, G.J., Katzman, R., and Agranoff, B.W. (Eds.): Basic Neurochemistry. Little, Brown and Co., 1972, pp. 191-206.

3. Gutensohn, W. and Guroff, G.: A rapid assay for purine phosphoribosyl transferases. Anal. Biochem. 47: 132-138, 1972.

4. Gutensohn, W. and Guroff, G.: Hypoxanthine-guanine phosphoribosyl-transferase from rat brain (purification, kinetic properties, development, and distribution). J. Neurochem. 19: 2139-2150, 1972.

5. DeLarco, J. and Guroff, G.: The synthesis of RNA which binds to oligo(dT)-cellulose by brain in vivo and in vitro. Biochem. Biophys. Res. Comm. 49: 1233-1240, 1972.

6. Nakagawa, S., and Guroff, G.: The uptake of purines by rat brain in vivo and in vitro. J. Neurochem., in press.

7. DeLarco, J. and Guroff, G.: The binding of RNA to various celluloses. Biochem. Biophys. Res. Comm. 50: 486-492, 1973.

Serial No. HD-LB4

1. Laboratory of Biomedical Sciences
2. Section on Physiological Controls
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Development of Mammalian Metabolic Regulation

Previous Serial Number: Same

Principal Investigator: W. H. Glinsmann

Other Investigators: H. Eisen
F. Zieve
M. Mitsunaga
F. Huang

Cooperating Units: I. Goldfine, Clinical Endocrinology Branch, NIAMD
K. Freude, Minneapolis Veterans Hospital, University of Minnesota
L. Zieve, Minneapolis Veterans Hospital, University of Minnesota
P. Sherline, Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

Man Years

Total:	69/12
Professional:	51/12
Others:	18/12

Project Description:

Objectives: This project seeks to define in mammalian cells: (1) mechanisms underlying differential modulation of carbohydrate and lipid metabolism and adaptive protein synthesis by hormones and nutrients; and (2) factors controlling development of these mechanisms. A general model is being examined based on control of protein phosphorylations which regulate rate-limiting enzymes. Current interest centers on a comprehensive description of glycogen cycle enzymes.

Methods Employed: Isolated cell and organ culture techniques and isolated organ perfusion are used in studying liver glucose production and rate-limiting enzymes in glycogen metabolism. Enzymes and regulatory proteins controlling glycogen turnover are isolated; their mode of regulation is described.

Major Findings: 1. Phosphoproteins controlling glycogen turnover: Many details of how cyclic AMP mediates the effects of glucagon and epinephrine

on glycogenolysis are known: cyclic AMP-activation of a general protein kinase which phosphorylates regulatory proteins has been studied extensively. However, increasing evidence supports the concept that hormonal and substrate effects on protein phosphorylation are also mediated through the corresponding phosphatase reactions. We are developing a detailed model for studying these interactions based on separation of glycogen cycle enzymes. Partial purification of the major enzymes has been achieved.

We postulated the existence of a general phosphoprotein phosphatase that reverses the effects of the cyclic AMP-dependent protein kinase and obtained initial evidence that glycogen synthetase phosphatase is this enzyme. Substrate specificity of the phosphatase, metal requirements, and effects of metabolic intermediates have been partially characterized.

Phosphorylase phosphatase, about which little is known, has been partially purified.

2. Development of hormonal and substrate regulation of glycogen turnover in isolated fetal liver: We have recently developed conditions for maintaining in defined media viable, hormonally-responsive explants of fetal and neonatal rat liver. The developmental pattern of glycogen synthesis has been duplicated in this system by the sequential administration of hydrocortisone and insulin. Evidence has been obtained for the following: (1) direct regulation of glycogen synthetase and phosphorylase by glucose does not occur in fetal tissue; (2) in term fetal explants, insulin stimulates glycogen synthesis and activation of glycogen synthetase by a process requiring protein synthesis; (3) at an early gestational age (day 15-16) when total glycogen synthetase levels are low, insulin does not stimulate glycogen synthesis although specific insulin receptors are fully developed; (4) the initial induction of glycogen synthetase requires hydrocortisone (glucocorticoids), and subsequent activation of this enzyme by insulin promotes glycogen synthesis.

The organ culture system in which the development of glycogen synthesis can be duplicated is likely applicable to studies in development of other regulatory systems. Additional studies with the system have shown that the fetal liver adrenergic receptor which controls glycogenolysis is of the beta type.

3. Post-heparin lipolytic enzymes: The hormonal stimulation of the hydrolysis of adipose tissue triglycerides has been studied extensively, and has been shown to be mediated through the cyclic AMP-dependent protein kinase. The opposite physiological process, i.e., the hydrolysis of plasma glycerides and their subsequent uptake by tissues, is also under hormonal control, but the molecular basis of this regulation has not yet been elucidated. As an initial step in the study of such regulation, we have separated and characterized the lipolytic activities of post-heparin plasma. Post-heparin monoglyceride esterase has been purified 10,000-fold and has been shown to be identical to post-heparin phospholipase A_1, but distinct from post-heparin lipase. Preliminary studies of the acute regulation of lipoprotein lipase in tissue extracts have been carried out. A rapid and sensitive lipase assay has been devised.

Significance to Biomedical Research and the Program of the Institute: Regulation of cellular metabolism, growth, and differentiation at many phylogenetic levels is in part mediated by a complex series of interactions between glucose and factors which alter cyclic AMP metabolism. Currently, the best characterized model for cyclic AMP action in mammalian tissue (where cyclic AMP acts as an intracellular mediator for many hormones and neurotransmitters) is the cascade of glycogen cycle protein phosphorylations initiated by cyclic AMP. We and others have shown that these reactions are also regulated by glucose, insulin, and glucocorticoids. Therefore a comprehensive decription of these reactions will hopefully allow us to develop a general model for cyclic AMP-glucose interactions in mammalian cells and thus provide a basis for detailed studies on factors affecting the development of the corresponding regulatory systems. The control of lipid metabolism is another system in which protein phosphorylations play an important regulatory role and in which interactions among hormones, substrates, and cyclic AMP can be studied.

Proposed Course of Project: Separation and characterization of enzymes participating in glycogen cycle phosphorylations will continue with particular emphasis on the regulatory roles of a general phosphoprotein phosphatase and phosphorylase phosphatase. Possible mechanisms mediating the effects of glucose and insulin will be sought.

Organ culture of fetal explants will be used to further define factors influencing the development of rate-limiting enzymes or hormone receptors that control glucose homeostasis, glycogen metabolism, gluconeogenesis, and cyclic AMP turnover.

The purification and characterization of the enzymes invovlved in the transport of fats from plasma into tissues will be continued. Immunologic techniques will be used to study the specific roles of the various enzymes in the hydrolysis of lipoprotein glycerides. The study of the acute regulation of plasma and tissue lipolytic enzymes will be pursued, with emphasis on the role of protein phosphorylations and dephosphorylations.

Honors and Awards: None

Publication:

1. Sherline, P., Lynch, A., and Glinsmann, W.: Cyclic AMP and adrenergic receptor control of rat liver glycogen metabolism. Endocrinology 91: 680-690, 1972.

2. Goldfine, I.D. and Sherline, P.: Insulin action in isolated rat thymocytes. II. independence of insulin and cyclic adenosine monophosphate. J. Biol. Chem. 247: 6927-6931, 1972.

3. Eisen, H.J. and Glinsmann, W.H.: Effects of insulin on glycogen synthesis in fetal rat liver in organ culture. Endocrinology 92: 584-588, 1973.

4. Zieve, F.J. and Glinsmann, W.H.: Activation of glycogen synthetase and inactivation of phosphorylase kinase by the same phosphoprotein phosphatase. Biochem. Biophys. Res. Commun. 50: 872-878, 1973.

5. Eisen, H.J., Sherline, P., and Glinsmann, W.H.: Activation of glycogen synthetase by insulin in fetal rat liver in organ culture. Endocrinology, in press.

Serial No. HD LB18

1. Laboratory of Biomedical Sciences
2. Section on Physiological Controls
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Regulation of Neuroendocrine Metabolism

Previous Serial Number: Same

Principal Investigator: D. C. Klein

Other Investigators: S. A. Binkley
A. Parfitt
M. A. Vaughan

Cooperating Units: R. Y. Moore, Division of Neurology, University of Chicago Medical School, Chicago, Illinois
R. Bensinger, Laboratory of Vision Research, NEI
A. Yuwiler and A. Geller, Neurobiochemistry Laboratory, V.A. Brentwood Hospital, Los Angeles, California
G. Jaim-Etchevery, Instituto de Anatomia General y Embriologia, Universidad de Buenos Aires, Argentina
L. Wetterberg and M. Backstrom, Uppsala University, Uppsala, Sweden
J. Pisano, Experimental Thrapeutic Branch, NHLI
S. MacBride and C. Ralph, Biology Department, University of Pittsburg, Pennsylvania

Man Years

Total:	60/12
Professional:	42/12
Others:	18/12

Project Description:

Objectives: (1) To describe the mechanism through which neuronal activity regulates biochemical activity in a neuroendocrine tissue, the pineal gland, and in the brain; (2) to describe factors regulating the development of enzyme systems and regulatory mechanisms in neural and neuroendocrine tissues; (3) to describe the role of N-acetylation of serotonin in the brain; (4) to describe the effects of drugs on indoleamine metabolism in developing and mature neuroendocrine tissue; (5) to isolate and characterize a pineal peptide which inhibits ovarian function; (6) to describe the regulation of the secretion and production of the antigonadotrophic pineal peptide.

Methods Employed: (1) Organ culture is used to provide a well defined and easily controlled experimental system for studying neurochemical regulation. Rat, human, and avian pineal tissue is cultured and treated with drugs and neurochemicals. The effects of these treatments are determined by measuring: (a) the total amount of tryptophan hydroxylated; (b) the amount of cyclic AMP in the tissue; (c) the activity of the enzyme that degrades cyclic AMP, phosphodiesterase; (d) the activity of N-acetyltransferase, the enzyme that converts serotonin to N-acetylserotonin; (e) the amount of serotonin in the gland and culture medium; and (f) the conversion of tryptophan to serotonin N-acetylserotonin, melatonin, hydroxyindoleacetic acid, hydroxtryptophol, and other serotonin derivatives. (2) The development of enzyme systems and regulatory mechanism in neural tissue is studied by describing the development of a system in the intact animal. Two systems of interest are the regulatory system controlling the activity of N-acetyltransferase and the activity of hydroxyindole-O-methyltransferase. The biochemically undifferentiated tissue is cultured and treated with hormones, transmitters, and drugs to study the factors regulating the biochemical differentiation of the tissue. (3) The pineal peptide is isolated by classical biochemical techniques including organic partitioning, gel filtration and thin-layer chromatography. The active material is monitored using the compensatory ovarian hypertrophy assay. The purified material is analyzed with an amino acid analyzer.

Major Findings: 1. Neural regulation of biochemical activity: a. Adrenergic regulation of serotonin: The amount of serotonin in the pineal gland is regulated by the release of norepinephrine from nerve endings. The released transmitter interacts with highly specific receptors in postsynaptic membranes. This receptor responds to the L-form of norepinephrine but not the D-form, does not respond to dopamine, and can be blocked by a β-adrenergic antagonist but not by α-adrenergic antagonists. The β-adrenergic protagonist, isoprel, mimicks the effects of norepinephrine, but the α-adrenergic antagonist, phenylephrine, is without effect. The effects of the interaction of norepinephrine with the receptor is to increase the activity of adenyl cyclase. This in turn causes an increase in the amount of cyclic AMP in the cells. The increase in the amount of cyclic AMP causes an increase in the activity of N-acetyltransferase, resulting in a decrease in the amount of serotonin in the cell and an increase in the amount of N-acetylserotonin and melatonin produced. b. Regulation of tryptophan hydroxylation: The amount of tryptophan hydroxylated by a pineal gland is proportional to the concentration of external tryptophan up to 0.5 mM. At higher concentrations the rate of tryptophan hydroxylation gradually decreases. This decrease may be due to an accumulation of hydroxytryptophan or serotonin in the tissue. Hydroxylation depends upon ongoing protein synthesis; in the absence of protein synthesis, hydroxylation ceases after eight hours. c. Regulation of indoleamine metabolism in the pineal gland: The regulation of indoleamine metabolism in the avian pineal gland resembles that in the mammalian pineal gland. At night the activity of N-acetyltransferase increases many fold. This causes an increase in the amount of melatonin in the gland. The activity of hydroxyindole-O-methyl transferase, in contrast, does not increase. Unlike the rat pineal gland, the rhythms in indoleamine metabolism in the bird persist in the absence of the superior cervical ganglia.

2. Pineal peptides: A peptide has been isolated that has a molecular weight less than 2000. Analysis of thin-layer chromatography and by N-terminal

dansylation indicates the preparation purified is homogenous. Less than 1 nanogram injected I.P. to a mouse 30 minutes after unilateral ovarectomy prevents the normal compensatory hypertrophy usually observed 6 days later.

3. Regulation of biological rhythms: The circadian rhythm in N-acetyltransferase appears to be driven by an endogenously generated "clock" in the suprachiasmatic nucleus. Destruction of all apparent neural input to this area does not block the circadian rhythm, whereas sectioning the neural output from this area does.

Significance to Biomedical Research and the Program of the Institute: These studies have provided explanations of how neurochemical metabolism can be regulated. Such explanations may also apply to neural tissue in general and provide models for describing brain function. The studies with drugs will also explain some of the effects of drugs on a molecular basis and will allow for a better understanding of the action of drugs and the normal functioning of neural tissue and for the development of more efficient drugs in the future. The pineal peptide that has been isolated will provide the reproductive endocrinologist with another valuable tool that he may use as a drug or as a diagnostic tool. This compound could be used effectively in the regulation of reproduction because it is so potent and long acting.

Proposed Course of Project: (1) The regulation of neurochemical metabolism will have as its focus the regulation of the activity of N-acetyltransferase. The nature of the neural control of gene expression will be determined. (2) The effects of hormones and drugs on the neural control of gene expression will be investigated. The nature of the regulation of biochemical differentiation will be studied. (3) The pineal peptide will be described as to amino acid composition and sequence. The peptide will be synthesized and the effects of the peptide studied. A radioimmunoassay for the peptide will be developed and the physiology of the production and secretion of the peptide will be described.

Honors and Awards: Neurosciences Research Program Fellow, Intensive Study Program, 1972.

Publications:

1. Berg, G.R. and Klein, D.C.: Norepinephrine stimulates ^{32}P incorporation into phosphatidyl inositol in membranes of cultured pineal glands. J. Neurochem. 19: 2519-2532, 1972.

2. Ellison, N., Weller, J., and Klein, D.C.: Development of a circadian rhythm in the activity of pineal serotonin N-acetyltransferase. J. Neurochem. 19: 1335-1341, 1972.

3. Silberstein, S.D., Lemberger, L., Klein, D.C., Axelrod, J., and Kopin, I.J.: Induction of adrenal tyrosine hydroxylase activity in organ culture. Neuropharmacology 11: 721-726, 1972.

4. Klein, D.C.: Evidence for the placental transfer of melatonin [acetyl-^{3}H]. Nature 237: 117-118, 1972.

5. Strada, S.J., Klein, D.C., Weller, J., and Weiss, B.: Effects of norepinephrine on the concentration of adenosine 3',5'-monophosphate of rat pineal gland in organ culture. Endocrinology 90: 1470-1475, 1972.

6. Klein, D.C.: The role of serotonin N-acetyltransferase in the adrenergic regulation of indole metabolism in the pineal gland. In Usdin, E. and Barchas, J. (Eds.): Serotonin and Behavior. New York, Academic Press, in press.

7. Klein, D.C. and Weller, J.L.: Rapid light-induced decrease in pineal serotonin N-acetyltransferase activity. Science 177: 532-533, 1972.

8. Klein, D.C.: Melatonin metabolism. In Kopin, I. and Rall, E. (Eds.): Methods in Investigative and Diagnostic Endocrinology. Amsterdam, North-Holland Publishing Co., 1972, pp. 550-568.

9. Klein, D.C. and Weller, J.: Adrenergic-adenosine 3',5'-monophosphate regulation of serotonin N-acetyltransferase activity and the temporal relationship of serotonin N-acetyltransferase activity to synthesis of ^{3}H-N-acetylserotonin and ^{3}H-melatonin in the cultured rat pineal gland. J. Pharm. Therap., in press.

10. Klein, D.C., Yuwiler, A., Weller, J.L., and Plotkin, S.: Post-synaptic adrenergic-cyclic AMP control of the serotonin content of cultured rat pineal glands. J. Neurochem., in press.

11. Klein, D.C.: Circadian rhythms in indole metabolism in the rat pineal gland. In Parker, D.K.O. (Ed.): The Neurosciences: Third Study Volume. Cambridge, MIT Press, in press.

12. Binkley, S., MacBride, S., Klein, D.C., and Ralph, C.: Pineal enzymes: Regulation of avian melatonin synthesis. Science, in press.

13. Coyle, J., Jacobowitz, S., Klein, D.C., and Axelrod, J.: Dopamine neurons in explants of substantia nigra in culture. J. Neurobiol., in press.

Serial No. HD-LB8(1)

1. Laboratory of Biomedical Sciences
2. Section on Developmental Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Developmental Pharmacology

Previous Serial Number: Same

Principal Investigator: D. W. Nebert

Other Investigators:
I. S. Owens
J. R. Robinson
A. Niwa
J. S. Felton
N. Considine
W. Tenney

Cooperating Units:
R. M. Friedman, Laboratory of Pathology, NCI
E. B. Thompson, Laboratory of Biochemistry, NCI
J. W. Daly, Laboratory of Chemistry, NIAMD
H. Kon, Laboratory of Chemical Physics, NIAMD
M. J. Coon, University of Michigan, Ann Arbor
A. P. Poland, University of Rochester Med. Sch., N.Y.
R. E. Kouri, Microbiological Associates, Inc., Bethesda
B. Paul, Birth Defects Institute of New York State, Albany

Man Years

Total	56/12
Professional	42/12
Others:	14/12

Project Description:

Objectives: Membrane-bound mixed-function oxygenases are important in the metabolism of drugs, carcinogens, and insecticides, as well as many endogenous lipophilic substrates such as steroids, hemin, and bilirubin. We found that one such oxygenase, aryl hydrocarbon hydroxylase, is inducible in mammalian fetal liver cells grown in culture by polycyclic hydrocarbons and by phenobarbital. Whereas it is well known that polycyclic hydrocarbons and phenobarbital induce hepatic drug-metabolizing enzymes by different mechanisms, the precise effects evoked by these two classes of microsomal enzyme inducers are not understood. Cell culture thus provides an opportunity for studying the mechanisms of microsomal oxygenase induction in an experimental system which is more rigidly controlled than similar studies in the intact animal.

Understanding the mechanisms by which drug-metabolizing enzymes are induced may elucidate important concepts in drug metabolism, developmental pharmacology, steroid biochemistry, chemical carcinogenesis, and entomology. The main objectives of this project are: (a) to compare the rates of hydroxylase synthesis and degradation and the physical properties of the phenobarbital-induced enzyme with those of the hydroxylase induced by polycyclic hydrocarbons, (b) to determine the extent to which hormones and nutrition affect the hydroxylase induction by either compound, (c) to characterize specific changes in nuclear acidic proteins, cellular RNA, and microsomal membrane protein during the induction process by either compound, (d) to measure entry and binding of various inducers to specific receptor sites within the hepatocytes, (e) to develop methods for inhibiting or stimulating the sequence of events involved during the oxygenase induction by either class of inducers, and (f) to use this experimental system for studying the control of specific gene expression in higher organisms.

Methods Employed: The principal methods employed are: (1) recording spectrophotometry of turbid solutions, (2) enzyme assays involving radiometric, spectrophotometric, and spectrophotofluorometric determinations of product formation or substrate disappearance, (3) radioisotopic measurements using tritium and carbon-14, (4) cell culture techniques, (5) differential centrifugation, (6) phase microscopy, (7) equilibrium dialysis, (8) polyacrylamide gel electrophoresis, (9) column and thin-layer chromatography, and (10) magnetic resonance techniques.

Major Findings: (1) Mouse skin tumorigenesis initiated by 7,12-dimethylbenz[a]anthracene or benzo[a]pyrene, with or without promotion by repeated applications of phorbol ester, is unrelated to genetic differences in the extent of aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons in inbred or hybrid C57BL/6N and DBA/2N mice; on the contrary, mouse sarcomas initiated by a single subcutaneous dose of 3-methylcholanthrene is related to the extent of the hydroxylase induction in these parent strains and their progeny from backcrosses and intercrosses. (2) Levels of hepatic epoxide hydrase activity were not closely associated with genetic differences in aryl hydrocarbon hydroxylase in these strains. (3) Aryl hydrocarbon hydroxylase activity is inducible in mouse 3T3 fibroblasts by benz[a]anthracene, whereas no detectable basal or inducible levels occur in hamster BHK cells or rat-hepatoma HTC cells and barely detectable inducible levels of this enzyme are found in human D98 cultures. Induction of the hydroxylase activity occurs to about the same degree as that in the mouse parent line--in 6 out of 9 mouse-hamster hybrids and in 3 out of 3 mouse-human hybrids formed by somatic-cell fusion. (4) In mouse 3T3-rat HTC hybrids, levels of inducible hydroxylase activity range from the same as to more than 20-fold greater than that in the 3T3 parent. (5) Tyrosine aminotransferase activity is inducible in HTC cells by dexamethasone, whereas only low noninducible levels of this enzyme exist in 3T3 cells; the aminotransferase levels remain very low and noninducible in all of these same fused somatic-cell hybrids, and there is immunological evidence indicating that all of the basal noninducible aminotransferase activity is derived from the mouse 3T3 parent. (6) Aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons is expressed dominantly, codominantly, or repressed dominantly, depending on the inbred strains of mice crossed.

(7) Induction of the microsomal hydroxylase activity by aromatic hydrocarbons is associated with concomitant synthesis of a new CO-binding hemoprotein, "cytochrome P-448," as determined by (i) spectral changes in the binding of n-octylamine to the microsomes, (ii) preferential inhibition of benzo[a]pyrene hydroxylation in vitro by various lipophilic test compounds, and (iii) paramagnetic changes in oxidized microsomes measured by electron paramagnetic resonance spectroscopy below 10° K; these changes are quite similar in liver or kidney microsomes from mouse, rat, or rabbit. (8) The rate at which the hydroxylase activity accumulates in fetal rat liver primary cultures treated with 1 mM norepinephrine or other biogenic amines of similar structure is quite similar to that by phenobarbital or polycyclic hydrocarbons. (9) The effect of norepinephrine is greater than that of phenobarbital or 3-methylcholanthrene in retarding the normal rate of degradation of induced hydroxylase activity in the presence or the absence of protein synthesis. (10) Either phenobarbital or 3-methylcholanthrene or norepinephrine as a second inducer can direct at some posttranscriptional level a further rise in hydroxylase activity after treatment of fetal rat hepatocyte cultures with the first inducer. (11) A simple radiometric assay for determining aminopyrine N-demethylase activity was developed, using the principle of partitioning the polar ^{14}C-formaldehyde metabolite complexed with semicarbazide from the lipophilic nonmetabolized substrate (methyl-^{14}C-labeled aminopyrine). This radioisotopic assay correlates well with the standard colorimetric method and is at least 100 times more sensitive. (12) In cell cultures previously treated with homologous interferon, the magnitude of antiviral activity and the degree of stimulation of aryl hydrocarbon hydroxylase induction appear to be directly related; both activities demonstrate species specificity, sensitivity to heat, and sensitivity to trypsin. (13) When mice are administered aromatic hydrocarbons, the induction of numerous enzyme "activities"--all membrane-bound mono-oxygenases having cytochrome P-450 associated with their active sites--is associated with the same genetic locus or with closely linked loci; these enzyme "activities" are: aryl hydrocarbon hydroxylase, p-nitroanisole O-demethylase, 7-ethoxycoumarin O-deethylase, 3-methyl-4-methylaminoazobenzene N-demethylase, zoxazolamine hydroxylase, acetanilide hydroxylase, naphthalene hydroxylase, and benzenesulfonanilide hydroxylase. (14) The expression of numerous other microsomal enzymes are not associated with this same genetic locus: aminopyrine N-demethylase, NADPH-cytochrome P-450 reductase, NADPH-cytochrome c reductase, d-benzphetamine N-demethylase, chlorcyclizine N-demethylase, ethylmorphine N-demethylase, pentobarbital hydroxylase, and 6β-, 7α-, and 16α-testosterone hydroxylases. (15) The extent of aryl hydrocarbon hydroxylase induction in various tissue of 10 inbred strains of mice by aromatic hydrocarbon administration in vivo is correlated with the magnitude of hydroxylase induction in fetal cell cultures derived from each of these strains. (16) Cytotoxicity in cell culture, caused by the metabolic potentiation of polycylic hydrocarbons by the inducible hydroxylase, is correlated with a decreased survival time when aromatic hydrocarbon-"responsive" mice are given large doses of polycyclic hydrocarbons. (17) Aryl hydrocarbon hydroxylase activity also is inducible in more than 5 liver-derived established cell lines treated with polycyclic hydrocarbons, biogenic amines, or phenobarbital in the growth medium.

Significance to Biomedical Research and the Program of the Institute: Phenobarbital is the most commonly used drug from a class of more than 200 drugs, chemicals, and insecticides that induce microsomal enzymes apparently by the same mechanism. Administration of any of these substances to the pregnant animal causes transplacental induction of these mixed-function oxygenases in the fetal liver. These enzyme systems metabolize steroids, hemin, cholesterol, fatty acids, indoles, sympathomimetic amines, thyroxine, and bilirubin, as well as lipophilic foreign substrates. Administration of polycyclic hydrocarbons to the pregnant animal produces induction of this enzyme activity in the placenta and also transplacental stimulation of the hydroxylase in several fetal tissues. Cigarette smoking is associated with increases in human placental aryl hydrocarbon hydroxylase activity. Consequently, studies involving the development and extent of induction of mixed-function oxygenases in fetal tissues and in the placenta during various periods of gestation, are relevant to the program of this Institute in the following areas: (1) possible effects of environmental pollutants (i.e., polycyclic hydrocarbons and insecticides) and various drugs on normal fetal growth and development and on the health of the pregnant woman, (2) mechanisms of "pharmacological immaturity" of the premature and newborn, (3) mechanisms of disease of the fetus and newborn concerning oxygenative and conjugative metabolism of normal body substrates such as steroids and bilirubin, and (4) possible therapeutic and diganostic procedures for aiding and predicting pharmacogenetic defects in the fetus and neonate.

Proposed Course of Project: (1) Further delineation of differences between mixed-function oxygenase induction by phenobarbital and that by polycyclic hydrocarbons, (2) characterization of specific and nonspecific binding of these various inducers to subcellular sites, (3) examination of the effects of hormones and nutrition on hydroxylase induction by either type of inducers, (4) study of various inhibitors on the induction process in cell culture, (5) characterization of the various components of the membrane-bound "hydroxylase" system, (6) analysis of differences in product formation and biophysical properties between the control, phenobarbital, and polycyclic hydrocarbon-induced oxygenases, and (7) extrapolation of these studies to formulate a general theory for explaining the mechanisms by which gene expression is controlled in mammalian cells.

Honors and Awards:

Nominated for John J. Abel Award, American Society for Pharmacology and Experimental Therapeutics. Nominated for Outstanding Young Scientist of Maryland Award

Publications:

1. Nebert, D.W., Benedict, W.F., Gielen, J.E., Oesch, F., and Daly, J.W.: Aryl hydrocarbon hydroxylase, epoxide hydrase, and 7,12-dimethylbenz[a]anthracene-produced skin tumorigenesis in the mouse. Mol. Pharmacol. 8: 374-379, 1972.

2. Nebert, D.W., and Gielen, J.E.: Genetic regulation of aryl hydrocarbon hydroxylase induction. Fed. Proc. 31: 1315-1325, 1972.

3. Benedict, W.F., Paul, B., and Nebert, D.W.: Expression of benz[a]-anthracene-inducible aryl hydrocarbon hydroxylase activity in mouse-hamster and mouse-human somatic-cell hybrids. Biochem. Biophys. Res. Commun. 48: 293-298, 1972.

4. Benedict, W.F., Nebert, D.W., and Thompson, E.B.: Expression of aryl hydrocarbon hydroxylase induction and suppression of tyrosine amino-transferase induction in somatic-cell hybrids. Proc. Nat. Acad. Sci. U.S.A. 69: 2179-2183, 1972.

5. Nebert, D.W., Gielen, J.E., and Goujon, F.M.: Genetic expression of aryl hydrocarbon hydroxylase induction. III. Changes in the binding of n-octylamine to cytochrome P-450. Mol. Pharmacol. 8: 651-666, 1972.

6. Goujon, F.M., Nebert, D.W., and Gielen, J.E.: Genetic expression of aryl hydrocarbon hydroxylase induction. IV. Interaction of various compounds with different forms of cytochrome P-450 and the effect on benzo[a]pyrene metabolism in vitro. Mol. Pharmacol. 8: 667-680, 1972.

7. Gielen, J.E. and Nebert, D.W.: Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. III. Effects of various sera, hormones, biogenic amines, and other endogenous compounds on the enzyme activity. J. Biol. Chem. 247: 7591-7602, 1972.

8. Nebert, D.W. and Kon, H.: Genetic regulation of aryl hydrocarbon hydroxylase induction. V. Specific changes in spin state of cytochrome P_{450} from genetically responsive animals. J. Biol. Chem. 248: 169-178, 1973.

9. Poland, A.P. and Nebert, D.W.: A sensitive radiometric assay of aminopyrine N-demethylation. J. Pharmacol. Exper. Therap. 184: 269-277, 1973.

10. Nebert, D.W. and Friedman, R.M.: Stimulation of aryl hydrocarbon hydroxylase induction in cell cultures by interferon. J. Virol. 11: 193-197, 1973.

11. Nebert, D.W., Considine, N., and Kon, H.: Genetic differences in cytochrome P-450 during induction of mono-oxygenase activities. Drug Metab. Dispos., in press.

12. Benedict, W.F., Considine, N., and Nebert, D.W.: Genetic differences in aryl hydrocarbon hydroxylase induction and benzo[a]pyrene-produced tumorigenesis in the mouse. Mol. Pharmacol., in press.

13. Nebert, D.W.: Use of fetal cell culture as an experimental system for predicting drug metabolism in the intact animal. Clin. Pharmacol. Therap., in press.

14. Nebert, D.W., Considine, N., and Owens, I.S.: Genetic expression of aryl hydrocarbon hydroxylase induction. VI. Control of other aromatic hydrocarbon-inducible mono-oxygenase activities at or near the same genetic locus. Arch. Biochem. Biophys., in press.

15. Benedict, W.F., Gielen, J.E., Owens, I.S., Niwa, A., and Nebert, D.W.: Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. IV. Stimulation of the enzyme activity in established cell lines derived from rat or mouse hepatoma and from normal rat liver. Biochem. Pharmacol., in press.

16. Nebert, D.W., Benedict, W.F., and Kouri, R.E.: Aromatic hydrocarbon-produced tumorigenesis and the genetic differences in aryl hydrocarbon hydroxylase induction. In Ts'o, P.O. and Dipaolo, J.A. (Eds.): Model Studies in Chemical Carciongenesis, in press.

17. Nebert, D.W.: Genetic and environmental factors influencing placental and fetal metabolism of drugs. In Moghissi, K.S. (Ed.): The Placenta: Biological and Clinical Aspects, in press.

NICHD Annual Report
July 1, 1972 through June 30, 1973
Pregnancy Research Branch

SUMMARY

The Pregnancy Research Branch has pursued its mission by investigating certain aspects of biochemistry and physiology in normal and abnormal mammalian pregnancy. The animal models used are the pregnant Rhesus monkey, (Macaca mulatta), the pregnant baboon, (Papio papio), and the pregnant Dorset sheep. In many instances data that has been obtained equates to that which has been available from human pregnancy; this fact strengthens and supports the belief that extrapolation from these models is pertinent to human pregnancy.

There are two main groups in the branch. The group in steroid hormone biochemistry has continued to evaluate and use the baboon as a model for studies directed at the elucidation of the mechanisms regulating steroid hormone synthesis and metabolism during pregnancy. The studies have provided additional confirmation that this species is a useful model for estrogen synthesis during human pregnancy in several important respects. As is the case in human pregnancy, placental utilization of estrogen precursors from the maternal circulation appears to be impeded. Thus rate limiting steps in the placenta as well as variations in the supply of precursors may regulate estrogen production. Preliminary data indicate that maternal and fetal metabolism of cortisol, particularly with respect to the extent of conjugation, differs from that of the nonpregnant state; this has also been found to be true for humans. Placental mechanisms for regulation of estrogen production have been studied by examination of placental activity of aromatase and sulfatase in normal and complicated human pregnancies. These two enzymes appear to be largely independent of maternal disease. It seems that variations in their total activities does not provide a general mechanism for regulation of estrogen production; however, competitive inhibition of the sulfatase by endogenous steroids and in particular by alternative substrates may provide such a mechanism. Studies with synthetic sulfatase inhibitors may provide an opportunity for experimental modulation of estrogen synthesis.

Finally, a comparison has been made of the utility of estrogen determinations on maternal urine and serum for assessment of feto-placental status in the high risk human pregnancy. Despite certain practical and theoretical advantages claimed for blood determinations, this study showed that in most high risk pregnancies serial 24 hour urinary determinations provide the most reliable data to monitor estrogen metabolism as a criterion of feto-placental well being. An extension of this work has been the finding of a circadian rhythm in blood and urine estrogen concentrations in the third trimester. This has been attributed to changes in precursors known to be secreted rhythmically by the maternal adrenal.

The other group in the branch has concerned itself with the endocrine physiology that pertains in pregnancy. The ongoing study of carbohydrate metabolism during primate pregnancy has been further expanded through the availability of an accurate and sensitive radioimmunoassay for monkey glucagon. Glucagon

levels in the mother and fetus are not affected by fasting or induced hyperglycemia in either the normal or the streptozotocin induced glucose intolerant animal. Glucagon intravenously administered in physiologic amounts does not cross the monkey placenta in either direction. In the newborn, there is a linear relationship between plasma glucose and glucagon levels whereas it is not present in the fetus.

A new protocol to examine renin-angiotensin relationships has been started in sheep pregnancies. This animal model allows the chronic implantation of both maternal and fetal catheters for periods of up to three weeks and thereby allows the measurement of blood components obtained in a nonstressed, nonanesthetized state. Preliminary evidence on plasma renin activity suggests that the baseline plasma renin activities of the mother are relatively constant on a day to day basis but those of the fetus are not.

The other major approach of this group is examination of calcium homeostasis in the subhuman pregnant primate. The methodology has now been acquired to assay parathormone by radioimmunoassay, to measure ionized calcium with a flow through electrode, and to measure total calcium with atomic absorption spectrometry. Preliminary evidence indicates that parathormone cannot be detected in fetal plasma. When the fetal ionized calcium levels are decreased by the infusion of EDTA, barely detectable concentrations of parathormone are found.

Studies on the amniotic fluid fat composition of primate pregnancy have been completed this year. The pattern of amniotic fluid fat concentration changes near term in both the baboon and the monkey in a manner similar to that found in the human. The lecithin/sphingomyelin ratio abruptly reverses at 94% gestational age. In the monkey this is associated with an increasing proportion of palmitic acid esters and relatively fewer myristic acid esters as it is in the human. In contrast, no change in lecithin fatty acid composition occurs in the baboon at this time but rather both of the two pathways responsible for lecithin production increase activity in parallel. This indicates that the monkey is an acceptable model for examination of the physiology and pathophysiology of lung maturation in the human whereas the baboon is not.

The fact that fetal experiments in the subhuman primate must be performed under anesthesia markedly limits the versatility of this animal model. This year considerable time and effort has been directed toward the maintenance of a chronic preparation in the baboon. The approach has been primarily directed toward technologic improvements in existing restraining devices plus methodology to maintain catheter patency over long periods of time. The stress of restraint appears to be associated with the premature induction of labor. However, there does appear to be a time interval in mid-pregnancy in which the animal can be introduced to the restraint without the complication of labor occurring. It also appears that catheter patency can be maintained in both the carotid artery and in the jugular vein for periods of up to one month. The next important step will be to place catheters into fetal vessels, ameliorate initial postoperative uterine irritability, maintain catheter patency for periods greater than five days, and then perform physiologic experiments without upsetting maternal homeostasis. There is every reason to

believe that this will be an extremely difficult task but the reward deserves the effort.

The branch has been considerably strengthened by the addition of two more professionals, a move of the laboratories to the Clinical Center in which ease of communication and consultation is much greater, and the presence of an active building program. An animal operating suite has been constructed and will be ready for utilization soon. The major problem in the animal work has been the acquisition of accurately timed gestations. The problem is most acute in sheep and least acute in the monkey. It is anticipated that many of the problems will be eliminated by the fall.

The branch has been responsible for the care of three pregnant women who had concurrent medical diseases which were being managed by other groups in the Clinical Center. Two of these patients were unique in the medical literature. The care of the patients and their subsequent newborns have emphasized the appropriateness of acquiring additional facilities, personnel, and laboratory resources if a competent professional effort is to be expended for the care of pregnant humans. The beginning of construction of the clinical facility of this branch further emphasizes the need to attract competent clinicians with research interests oriented toward physiological and biochemical questions in human pregnancy. This will be a major activity of the branch this year.

Serial No. HD-PR1
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Carbohydrate Metabolism during Primate Pregnancy

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Alan R. Fleischman, M.D.; Daniel H. Mintz, M.D., University of Miami; Antoinette Schenk, University of Miami; Donald L. Hutchinson, M.D., University of Pittsburgh; I. Arthur Mirsky, M.D., VA Hospital, Los Angeles, California; Hernando Salazar, M.D., University of Pittsburgh; Rosemary Fox, University of Pittsburgh; Robert Miller; Sally Bowen, B.S.

Cooperating Units: Experimental Surgery & Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services, NIH.

Man Years:

Total:	4.5
Professional:	2.0
Other:	2.5

Project Description:

Objectives:

1. To examine the effect of oral glycemic stimuli on fetal pancreatic beta cell responsiveness.
2. To determine if glucagon is transferred across the primate placenta and if hyperglucagonemia exists in pregnancies associated with maternal carbohydrate intolerance.
3. To compare a monkey pregnant model of induced diabetes mellitus with data presently available from human pregnancies associated with diabetes mellitus.
4. To determine if there is limitation to maternal-fetal placental transfer of glucose.
5. To determine if streptozotocin is transferred to the fetus from the mother.

Methods Employed: Accurately timed gestations in Rhesus monkeys are used. Streptozotocin, 40-48 mg/kg, is administered intravenously to the adult female prior to or in the first trimester of pregnancy. This drug induces a glucose intolerant state secondary to pancreatic beta cell destruction. During the third trimester, acute experiments are performed under general

anesthesia and aseptic surgical techniques. Cannulae are placed into both the maternal and fetal circulations to permit sequential sampling of blood. A catheter is placed transuterine into the fetal stomach via the mouth and glucose solution is injected into the stomach lumen. Blood aliquots are analyzed for glucose, free fatty acids, insulin growth hormone, and glucagon using biochemical and radioimmunoassay procedures. After performing the same experiment on the newborn in the first day of life, tissue is examined histologically from both mother and conceptus.

Major Findings: The primary focus of this work has been in progress for several years. The results to date are summarized in four articles. J. Clin. Invest. 48:176, '69, J. Clin. Invest. 49:1517, '70, J. Clin. Invest. Metab. 20:805, '71, J. Clin. Invest. 51:837, '72. The work done this year has demonstrated a failure of glucose orally administered in utero to enhance plasma insulin concentrations in either the normal or abnormal fetus. When the same stimulus is administered to the newborn, plasma insulin levels increase. Glucagon, intravenously administered in physiologic amounts, does not cross the monkey placenta in either direction. There is a lack of hyperglucagonemia during fasting or induced hyperglycemia in the normal or streptozotocin mother or fetus. There is a linear relationship between newborn plasma glucose and glucagon levels. Streptozotocin crosses the monkey placenta in all stages of gestation. The rate of transfer and the dilution by maternal blood result in maximum fetal concentrations 1/10th that of the mother. No histological or anthropometric or biochemical effect of this reduced dose can be measured in the newborn.

Significance to Bio-Medical Research and to the Institute: Prior to this study, the interrelationships of fetal and maternal glucose, insulin, growth hormone, and glucagon could only be conjectured in primate pregnancy. The success of the streptozotocin diabetic model has validated the theory of fetal hyperinsulinism in diabetic pregnancies. In question is the singular significance of maternal euglycemia in the measurement of diabetic pregnancies if maternal hyperaminoacidemia coexists. The therapeutic implications for human pregnancies are primarily in the area of basic mechanisms for the early induction of fetal beta cell responsiveness to glycemic and amino acid stimuli prior to term delivery.

Proposed Course: The absence of a chronic fetal preparation in the monkey limits the direction of this protocol. Until such time as one is developed, no new protocols in pregnancy are being planned.

The effects of this induced carbohydrate intolerance state on adult monkey retina and muscle capillary basement membranes are now being investigated.

Honors and Awards: Ronald A. Chez

Appointed Clinical Professor, Department Ob-Gyn, Howard University School of Medicine, Washington, D.C., 1972.
Member, Committee on Nutrition, American College of Obstetrics and Gynecologists, 1972.
Member, Committee for Annual Clinical Meeting, American College of Obstetrics and Gynecologists, 1972.

Member, Editorial Board, Contemporary Ob-Gyn, 1972.
Member, Editorial Board, MedCom, 1972.
Member, Editorial Board, Human Reproduction, 1973.
Member, Ad Hoc Panel on Postcoital Estrogens (Center for Population, NICHD), January, 1973.
Member, FIC Committee on Prevention of Fetal and Perinatal Diseases, 1973.
Invited Speaker, The Institute for Comprehensive Medicine, Crystal City, Virginia, June, 1972.
Invited Lecturer, Sir Joseph Barcroft Centenary Symposium, Cambridge, England, July, 1972.
Invited Lecturer, New York University Graduate Program, New York, New York, September, 1972.
Invited Lecturer, Family Planning Council of Southwestern Pa. & the Commonwealth of Pennsylvania, Pittsburgh, Pa., October, 1972.
Invited Lecturer, Howard University, Washington, D.C., November, 1972.
Invited Lecturer, D.C. Society of Obstetricians and Gynecologists, Washington, D.C., December, 1972.
Invited Lecturer, Columbia Hospital for Women, Washington, D.C., January, 1973.
Invited Speaker, Ross Conference on "The Endocrine Milieu of Pregnancy, Puerperium and Childhood", St. Maarten, February, 1973.
Invited Speaker, The National Foundation-March of Dimes, New York, New York, March, 1973.
Invited Lecturer, Obstetrical Society of Northern Virginia, Seven Corners, Virginia, March, 1973.
Invited Lecturer, Harvard University Medical School, Boston, Mass., March, 1973.
Invited Lecturer, Washington Adventist Hospital, Takoma Park, Maryland, April, 1973.
Invited Lecturer, The University of Iowa Hospitals & Clinics, Iowa City, Iowa, April, 1973.
Invited Lecturer, George Washington University Hospital, Washington, D.C., May, 1973.
Visiting Professor, University of Hawaii, Honolulu, Hawaii, May, 1973.
Invited Speaker, Fairfax Hospital, Falls Church, Virginia, June, 1973.

Publications:

Mintz, D.H., Chez, R.A., Hutchinson, D.L.: Subhuman Primate Pregnancy Complicated By Streptozotocin-Induced Diabetes Mellitus., J. Clin. Invest. 51:837, 1972.

Chez, R.A., Mintz, D.H., Hutchinson, D.L.; Horger, E.O. III.: Third Trimester Simian Fetal Carbohydrate Metabolism., In Physiological Biochemistry of the Fetus, Hodari, Charles C. Thomas, Springfield, 1972.

Chez, R.A., Josimovich, J.B., Schultz, S.G.: The Transfer of HPL Across Isolated Amnion-Chorion., Gynec. Invest. 1:312, 1972.

Chez, R.A.: In Vitro Flux of Human Placental Lactogen Across Fetal Membranes., In 6th Rochester Trophoblast Conference, 1971, Lund and Choate, University of Rochester Press, 1972.

Chez, R.A., Schlesselman, J.J., Salazar, H., Fox, R.: Single Placentas in the Rhesus Monkey., J. Med. Prim. 1:230, 1972.

Chez, R.A., Mintz, D.H.: The Development and Function of the Human Endocrine Pancreas., In Symposium "The Endocrine Milieu of Pregnancy, Puerperium and Childhood." Ross Laboratories, Columbus, Ohio, 1973.

Chez, R.A.: Fetal Factors Relating to Labor, In Uterine Contraction - Side Effects of Steroidal Contraceptives, Josimovich, Wiley-Interscience, New York, 1973.

Salazar, H., Chez, R.A., Pardo, M.: Absence of Ultrastructural Changes in the Basement Membrane of Monkey Muscle Capillaries in Streptozotocin - Induced Carbohydrate Intolerance., Amer. J. Patho., 1973, (In Press).

Chez, R.A., Mintz, D.H., Hutchinson, D.L.: Carbohydrate Metabolism in Primate Pregnancy., In Proc. 4th Int'l Congress on Endocrinology, Excerpta Medica, 1973, (In Press).

Chez, R.A.: The Role of the Pharmacist in Family Planning: A Pennsylvania Survey., J. Amer. Pharm. Assoc.NS12:464, 1972.

Andrews, B.F., Apgar, V., Chez, R.A., Freeman, M.G., Gluck, L., Kubarych, S., Queenan, J.T., Warrick, L.: New Management Concepts in High-Risk Pregnancy, Patient Care: 96-141, March 15, 1973.

Andrews, B.F., Apgar, V., Chez, R.A., Freeman, M.G., Gluck, L., Queenan, J.T., Warrick, L.: Identifying High-Risk Pregnancy - In Time., Patient Care: 18-50, September 30, 1972.

Chez, R.A.: Prenatal Care - "First Do No Harm.", Contemp. Ob/Gyn 1:9, 1973.

Chez, R.A., Fleischman, A.R.: Fetal Therapeutics - Challenges and Responsibilities., Clin. Pharm. and Therapeutics, 1973 (In Press).

Chez, R.A.: Drugs and the Unborn Patient., Contemp. Ob/Gyn, 1973 (In Press).

Chez, R.A.: The Use of Learning Aids in a Human Sterilization Program., In Human Sterilization, Richart and Prager, Charles C. Thomas, Springfield, 1972.

Brackin, M.A., Chez, R.A.: A Programmed Audiovisual Orientation to an Outpatient Clinic., J.Obstet.Gynec.& Neonatal Nursing, 1:33, 1972.

Stenchever, M.A., Chez, R.A., et al.: The APGO Steering Committee for Cooperative Teaching in Obstetrics and Gynecology-National Library of Medicine Project-A Progress Report., J. Reprod. Med. 10:19, 1973.

Serial No. HD-PR2
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Steroid Hormones in Primate Pregnancy

Principal Investigator: John D. Townsley, Ph.D.

Other Investigators: C. Deans Crystle, M.D., Gerald J. Pepe, Ph.D., Nancy M. Jensen, B.S., Elliece Smith, B.S., Linda J. Gartman, Norman Dubin, Ph.D., University of Maryland; George Grannis, Ph.D., Ohio State University.

Cooperating Units: Experimental Surgery & Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services.

Man Years:

Total:	5.2
Professional:	3.2
Other:	2.0

Project Description:

Objectives:

1. To ellucidate the mechanisms regulating steroid hormone synthesis and metabolism during human pregnancy.
2. To evaluate and use the baboon as a model for such studies.
3. To compare the utility of estrogen determinations in maternal urine and serum for the assessment of feto-placental status in the high-risk pregnancy.

Methods Employed: The project demands a variety of complementary approaches ranging from metabolic studies in the intact pregnant primate to determination of activities of steroidogenic enzymes in sub-cellular fractions of placenta. Analytical methods include fluorometric, colorimetric, and radioimmunoassays for steroids in body fluids and double isotope techniques for kinetic enzyme assays and the metabolic studies in vivo.

Major Findings: Comparative evaluation of serum estradiol-17β and estriol and urinary total estrogens and estrogen-creatinine ratio as indices of fetal health has been completed. Despite certain practical and theoretical advantages claimed for blood determinations this study showed that in most high-risk pregnancies serial 24 hr urinary determinations provide the most reliable data to monitor estrogen metabolism as one parameter of feto-placental well-being. The circadian rhythm in blood and urine estrogen concentrations, which we have shown in the third trimester and attributed

to changes in precursors known to be secreted rhythmically by the maternal adrenal, has subsequently been confirmed in two other laboratories.

Determination of human placental aromatase and sulfatase activities in normal and complicated pregnancies indicate that they are largely independent of maternal disease and that variations in their activity does not provide a general mechanism for regulation of estrogen production. However, competitive inhibition of the sulfatase by endogenous steroids, in particular alternative substrates, may well provide such a mechanism. Our studies indicate that synthetic inhibitors may be found which can be used for experimental modulation of estrogen synthesis. Estrogen precursors administrated intra-amniotically appear to be utilized more readily than those provided via the maternal circulation. This suggests that the importance of precursor availability in regulating placental estrogen production may depend on the origin of the precursor.

Studies with pregnant baboons confirm our previous conclusion that this species provides a useful model for estrogen synthesis during human pregnancy in several important respects. As is the case in human pregnancy utilization of estrogen precursors from the maternal circulation appears to be impeded. Preliminary data indicate that maternal and fetal metabolism of cortisol, particularly with respect to the extent of conjugation, differs from that of the nonpregnant animal. This is also true for humans.

Significance to Bio-Medical Research and to the Institute: Because of the widespread utilization of steroid hormone assays to monitor feto-placental status and the apparent importance of such hormones in maintaining the pregnancy and in the onset of labor an understanding of the mechanisms controlling their synthesis and metabolism is essential for definitive resolution of many problems of reproductive failure. Our approaches provide fundamental knowledge of these processes and practical information of immediate use to the clinician. Further elucidation of controlling mechanisms will require manipulative procedures in both the mother and the fetus which are precluded in humans. An adequate animal model must be found in order to do this and our studies continue to indicate that the baboon is a promising candidate.

Proposed Course: Our major thrust will involve the baboon as a model. Studies indicating impeded utilization of maternal circulating estrogen precursors will be extended and the reasons for this finding elucidated. The importance of variation in fetal circulating precursors in regulating estrogen production will be explored. The intrauterine and neonatal maturation of the fetal-newborn adrenal-pituitary-hypothalmic axis, as judged by changing response to tropic stimuli and C_{19} and C_{21} steroid production, will be studied. Investigation of placental mechanisms regulating estrogen synthesis in the presence of excess precursors will be continued.

Honors and Awards: John D. Townsley

Invited Lecturer, Fifty Fifth Annual Meeting of the Endocrine Society, Chicago, June, 1973.

Publications:

Townsley, J.D.: Steroid Hormones in pregnancy: The Baboon as a model for Studies on Estrogen Synthesis., Acta Endocr. Suppl 166:191, 1972.

Townsley, J.D., Rubin, E.J.: Structure-Activity Correlations for Steroid Inhibition of Human Placental Steroid 3-Sulfatase., Research on Steroids, 1972, (In Press).

Townsley, J.D., Dubin, N.H., Grannis, G.F., Gartman, L.J., Crystle, C.D.: Circadian Rhythms of Serum and Urinary Estrogens in Pregnancy., J. Clin. Endocr.36:289, 1973.

Dubin, N.H., Crystle, C.D., Grannis, G.F., Townsley, J.D.: Comparison of Maternal Serum Estriol and Urinary Estrogen Determinations as Indices of Fetal Health.,Am. J. Ob. Gyn. 115:835, 1973.

Townsley, J.D., Gartman, L.J., Crystle, C.D.: Maternal Serum 17β-Estradiol Levels in Normal and Complicated Pregnancies: A Comparison with other Estrogen Indices of Fetal Health., Am. J. Ob. Gyn. 115:830, 1973.

Townsley, J.D.: Further Studies on the Regulation of Human Placental Steroid 3-Sulfatase Activity., Endocr. 1973, (In Press).

Townsley, J.D., Rubin, E.J., Crystle, C.D.: Evaluation of Placental Steroid 3-Sulfatase and Aromatase Activities as Regulators of Estrogen Production in Human Pregnancy., Am. J. Ob. Gyn. 1973, (In Press).

Honors and Awards: C. Deans Crystle

Appointed Assistant Clinical Professor, Department Obstetrics and Gynecology, Georgetown University, Washington, D.C., 1972.
Invited Lecturer, Ohio State University, Department Obstetrics and Gynecology, November, 1972.
Invited Lecturer, Good Samaritan Hospital, Department Obstetrics and Gynecology, Dayton, Ohio, November, 1972.
Invited Lecturer, University of Connecticut School of Medicine, Department Obstetrics and Gynecology, April, 1973.

Publications:

Townsley, J.D., Dubin, N.H., Grannis, G.F., Gartman, L.J., Crystle, C.D.: Circadian Rhythms of Serum and Urinary Estrogen in Pregnancy., J. Clin. Endocr. 36:289, 1973.

Townsley, J.D., Gartman, L.J., Crystle, C.D.: Maternal Serum 17β-Estradiol Levels in Normal and Complicated Pregnancies: A Comparison with other Estrogen Indices of Fetal Health., Am. J. Ob. Gyn. 115:830, 1973.

Dubin, N.H., Crystle, C.D., Grannis, G.F., Townsley, J.D.: Comparison of Maternal Serum Estriol and Urinary Estrogen Determinations as Indices of Fetal Health., Am. J. Ob. Gyn. 115:835, 1973.

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Honors and Awards: Gerald J. Pepe, None

Publications:

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Pepe, G.J., Rothchild, I.: Serum Progesterone Levels in Ovariectomized Rats Injected with Progesterone and Estrone: Relation to Pregnancy Maintenance and Growth of Decidual Tissue., Endocr. 1973, (In Press).

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Serial No. HD-PR3
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Amniotic Fluid Fat Concentrations in Primate Pregnancy

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Louis Gluck, M.D., University of California, La Jolla; Sally Bowen, B.S.

Cooperating Units: Experimental Surgery & Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services.

Man Years:

Total:	0.25
Professional:	0.10
Other:	0.15

Project Description:

Objectives:

1. To determine the patterns of amniotic fluid fat composition in monkey and baboon pregnancies.
2. To examine the effect of fetal tracheal and/or esophageal ligation on amniotic fluid phospholipid and sphingomyelin ratios.

Methods Employed: Rhesus monkeys (Macaca mulatta) and baboons (Papio papio) with accurately timed gestations are used. Transabdominal percutaneous amniocentesis is sequentially performed during pregnancy. Late in the second trimester, fetal tracheal and/or esophageal ligation is performed under aseptic surgical conditions. The fetus is delivered electively at term via cesarean section.

Major Findings: The pattern of amniotic fluid fat concentrations changes near term in both subhuman primate species in a manner similar to that found in the human. The lecithin sphingomyelin (L/S) abruptly reverses at 94% gestational age. In the monkey, this is associated with an increasing proportion of palmitic acid esters and relatively fewer myristic acid esters. In contrast, no change in lecithin fatty acid composition occurs in the baboon. Tracheal ligation is associated with nondistended lungs (in contrast to the sheep), and relative oligohydramnios. After surgery, the fatty acid esters on the surface active lecithin in the amniotic fluid did not change near term. Examination of the lecithin in the ligated bronchial tree-lungs showed it to have surface active leithin of fatty acid composition consistent with maturity and therefore with that in amniotic fluid. For reasons

that remain unclear, esophageal ligation is associated with early fetal demise.

Significance to Bio-Medical Research and to the Institute: The L/S in human amniotic fluid is a reliable diagnostic tool for the determination of fetal lung maturity. The effects of congenital anomalies, intrauterine stress, and maternal or fetal medication remain unknown. The use of this animal model will permit research into the physiology and pathophysiology of these pathways.

Proposed Course: An attempt to induce fetal lung maturation prior to its normal occurrence will be considered. There is a suggestion that intrauterine chronic asphyxia and fetal adrenal cortical activity are coregulators of fetal lung maturation. The effects of the administration of corticosteroids to the fetus and fetal adrenal-hypophysectomy may be examined.

It is possible to examine the kinetics of the 5 enzymes involved in lecithin formation in the lungs using in vitro lung slice incubation techniques. Fresh newborn lung is a byproduct of other experimental protocols. This protocol is presently being examined for possibility.

Honors and Awards: None

Publications: None

Serial No. HD-PR4
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Effects of Induced Fetal Blood pH changes on Fetal Blood Flow

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Alan R. Fleischman, M.D.; Andre Hellegers, M.D., Georgetown University; Robert Cefalo, M.D., Georgetown University; Joan Simkovich, Ph.D., Georgetown University; Sally Bowen, B.S.

Cooperating Units: Division of Research Services, Veterinary Resources Branch, Animal Center Section, Ungulate Unit, NIH

Man Years:

Total:	3.4
Professional:	3.0
Other:	0.4

Project Description:

Objectives:

1. To study the effects of induced fetal metabolic acidosis on fetal blood flow, fetal oxygen consumption, uterine blood flow, and fetal and maternal plasma oxygen tensions.
2. To study the effects of induced fetal respiratory acidosis on the same parameters.
3. To determine whether permeability characteristics of placental oxygen transfer can be modified.

Methods Employed: Accurately timed gestations in Dorset sheep are utilized. Under anesthesia and aseptic surgical conditions, cannulae are placed into the maternal and fetal circulations. The latter is done without interrupting the integrity of the amniotic sac. The experiment is then performed under this acute set of conditions on 3-7 days subsequently after the animal has recovered and is in a nonstressed nonanesthetized state. Measurements are made with the autoanalyzer, Van Slyke apparatus, and blood gas-pH equipment.

Hemihysterectomy was performed in 16 sheep prior to conception; 12 of these conceived and are presently pregnant. Hemihysterectomy immediately prior to the acute experiments cited above have been done in six instances.

Major Findings: The production of a metabolic alkalosis confined to the fetus has no appreciable effect on fetal oxygen consumption although it decreases the fetal PO_2. The latter is produced by a leftward shift in the oxygen dissociation curve. Since the quantity of oxygen crossing the placenta is unaltered, the theoretical benefits to the fetus by the increase in its blood oxygen affinity do not in fact seem to operate.

The infusion of diamox into the fetus is a useful tool in producing unilateral fetal respiratory acidosis by the trapping of carbon dioxide on the fetal side of the placenta.

The removal of a portion of the placental implantation sites prior to pregnancy, or of the placenta itself during pregnancy has no deleterious effects on fetal growth, providing it is not accompanied by shunting of blood through the ablated area. In this respect the placenta seems to bear some analogy to the lung. The data tend to confirm that blood flow, rather than placental diffusion-limitation is critical in maternal-fetal nutrient transfer.

Halothane may be a hazardous anesthetic to the fetus since it decreases uterine blood flow, umbilical blood flow and fetal oxygen consumption. Of these, uterine blood flow probably caused secondarily by a decrease in cardiac output, is the greater hazard.

Significance to Bio-Medical Research and to the Institute: The utility of fetal heart rate, acid base, and gas monitoring in human pregnancy continues to be debated. In addition to a lack of data on the significance of some of the changes observed, there is a lack of information as to fetal therapeutic implications. Data from the pregnant sheep model has been successfully extrapolated to human pregnancy in several areas. It is believed that this project will provide physiological and therapeutic information on fetal acid-base control mechanisms, fetal blood flow, and fetal oxygen consumption with and without anesthesia.

Proposed Course: No new protocols are planned at this time.

Honors and Awards: None

Publications: Ph.D. Thesis, Joan Simkovich

Serial No. HD-PR5
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Calcium Homeostasis in the Subhuman Pregnant Primate

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Alan R. Fleischman, M.D.; Daniel H. Mintz, M.D., University of Miami; Antoinette Schenk, University of Miami; Sally Bowen, B.S.

Cooperating Units: Experimental Surgery & Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services.

Man Years:

Total:	1.5
Professional:	0.6
Other:	0.9

Project Description:

Objectives:

1. To examine the bidirectional placental transfer of radioisotopically labelled bovine parathyroid hormone and synthetic human calcitonin.
2. To study the effects of perturbations of maternal and fetal serum calcium and phosphorous on the serum calcium, ionized calcium, and immunoreactive parathyroid hormone and calcitonin in the fetal and maternal circulations.
3. To study the effects of the intravascular administration of PTH and CT on placental transport of radiolabelled calcium.
4. To investigate the hormonal control of calcium homeostasis in the neonate and the pathophysiology associated with neonatal tetany.

Methods Employed: Rhesus monkeys (Macaca mulatta and Macaca speciosa) with accurately timed gestations are used. Under aseptic surgical conditions, cannulae are placed into the fetal and maternal circulations. An attempt is made to maintain the integrity of the amniotic sac. Highly purified bovine PTH or synthetic human CT iodinated with radioactive iodine, calcium gluconate, or EDTA are intravenously administered to mother or fetus. Blood aliquots are sequentially obtained for appropriate measurements utilizing atomic absorption spectrometry, flow through electrodes, and radioimmunoassay.

Major Findings: Preliminary data suggests that a limited bidirectional placental transfer of PTH occurs in the third trimester, and that CT does not cross the placenta. PTH cannot be detected in fetal plasma. When the

fetal ionized calcium levels are decreased by the infusion of EDTA, barely detectable concentrations are measured.

Significance to Bio-Medical Research and to the Institute: Pregnancy elicits a physiologic adaptive response in the mother to protect a late pregnancy fetal claim on calcium for mineralization of the fetal skeleton. There is a paucity of decisive data in human pregnancy secondary to the inherent experimental difficulties encountered in studying healthy patients and the lack until recently of reliable radioimmunoassay techniques for the measurement of plasma levels of parathormone and thyroid calcitonin. The utilization of the subhuman primate permits investigation of this complex problem with techniques which permit study of the fetus in utero and assay procedures that can be performed on small aliquots of plasma. It is hoped that the data generated will provide the first definitive exploration in primates of the parathyroid-calcitonin releasing mechanism studied simultaneously in both fetus and mother.

Proposed Course: All aspects of the project require exploration and a major commitment to this project is planned. The obtaining of data will depend upon the availability of pregnant monkeys now that the radioimmunoassay and ionized calcium techniques have been developed.

Honors and Awards: None

Publications: None

Serial No. HD-PR6
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Prolactin Metabolism During Primate Pregnancy

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Alan R. Fleischman, M.D.; John E.A. Tyson, M.D., Johns Hopkins University; Janice Huth, Johns Hopkins University; Beverly Smith, Johns Hopkins University; Prudence Thomas, Johns Hopkins University; Sally Bowen, B.S.

Cooperating Units: Experimental Surgery & Clinical Medicine Section, Laboratory Aids Branch, Division of Research Services.

Man Years:

Total:	2.7
Professional:	1.0
Other:	1.7

Project Description:

Objectives: To study the bidirectional placental and amniotic fluid transfer of prolactin in primate pregnancy.

Methods Employed: Ten Rhesus monkeys (Macaca mulatta and Macaca speciosa) with accurately timed gestations have been used. Under aseptic surgical conditions, cannulae are placed into the maternal and fetal circulations as well as the intact amniotic sac. Highly purified human prolactin iodinated with radioactive isotopes is injected into one of the three compartments. Sequential aliquots of blood and amniotic fluid are examined for hot and cold prolactin concentrations using radioimmunoassay and chromatography techniques.

Major Findings: Prolactin does not cross the primate placenta in either direction in the third trimester. The prolactin in amniotic fluid is only partially of maternal origin and not of fetal origin. Prolactin in amniotic fluid exchanges minimally with fetal or maternal blood.

Significance to Bio-Medical Research and to the Institute: The amniotic fluid concentration of prolactin is approximately ten times that of the levels found in maternal and fetal plasma. Prolactin has been shown to affect the transfer of sodium across the gills of fish and the renal tubules of dogs. The role of prolactin in the exchange of water and electrolytes across fetal membranes needs to be clarified. This study describes some of the fundamental physiology necessary as a foundation.

Proposed Course: Further use of the intact primate pregnancy is not planned. Under consideration is a protocol examining the effects of prolactin on sodium transfer across fetal membranes mounted in an Ussing chamber.

Honors and Awards: None

Publications: None

Serial No. HD-PR-7
1. Pregnancy Research Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Renin-Angiotensin Relationships in Sheep Pregnancy

Principal Investigator: Ronald A. Chez, M.D.

Other Investigators: Alan R. Fleischman, M.D.; Gary K. Oakes, M.D.; Kevin J. Catt, M.D., Reproduction Research Branch, NICHD; Martha Ashburn, Reproduction Research Branch, NICHD; Sally Bowen, B.S.

Cooperating Units: NIH Animal Center, Poolesville, Maryland

Man Years:

Total:	5.5
Professional:	4.0
Other:	0.2

Project Description:

Objectives:

1. To examine the bidirectional placental transfer of renin.
2. To study the effects of perturbations of maternal and fetal plasma electrolytes and volume on serum renin activity.

Methods Employed: Accurately timed gestations in Dorset sheep are utilized. Under anesthesia and aseptic surgical conditions, cannulae are placed into the maternal and fetal circulations. The latter is done without interrupting the integrity of the amniotic sac. The experiment is then performed under this acute set of conditions on 3-21 days subsequently after the animal has recovered and is in a nonstressed nonanesthetized state. Measurements of renin activity are made utilizing radioimmunoassay techniques.

Major Findings: The baseline plasma renin activity of the mother is constant from day to day in the third trimester; that of the fetus is not with marked daily fluctuations and a consistent positive gradient to the mother. Potent diuretics administered to the mother are associated with elevations in maternal plasma renin activity and a variable response in the fetus. Fetal nephrectomy at the beginning of the third trimester is accompanied by a high mortality for reasons that are not yet clear.

Significance to Bio-Medical Research and to the Institute: The Renin-Angiotensin system is a major determinant of salt and water metabolism in pregnancy. The factors and variables that regulate and influence the production and

secretion in pregnancy of renin are not known. The possibility exists that the system in the fetus and mother are interdependent or independent. The implications of such a relationship may have import in human prenatal care and the management of disease states in pregnancy.

Proposed Course: All aspects of the project require exploration and a major commitment to this project is planned.

Honors and Awards: None

Publications: None

PHS-NIH
Pregnancy Research Branch
Contract and Collaborative Research
July 1, 1972 through June 30, 1973

Contract Title: Human Fetal-Maternal Metabolism Studied Under Conditions of Acute Fasting

Principle Investigator: Ronald A. Chez, M.D.

Other Investigators: John E.A. Tyson, M. D., Johns Hopkins University; Janice Huth, John Hopkins University; Beverly Smith, Johns Hopkins University; Prudence Thomas, Johns Hopkins University.

Contractor: Johns Hopkins University
School of Medicine
Department of Obstetrics & Gynecology
Baltimore, Maryland 21205

Money Allocated: $103,305

Man Years:

Total:	4.2
Professional:	1.2
Other:	3.0

Contract Description:

Objectives:

1. To study the effects of acute starvation induced in the first half of pregnancy on human maternal plasma placental lactogen concentration.
2. To study the relationship between carbohydrate-insulin-growth hormone metabolism and human maternal plasma and amniotic fluid concentrations of prolactin.
3. To measure the concentrations of the essential and glucogenic amino acids in human maternal plasma, fetal plasma, and amniotic fluid under conditions of acute maternal starvation and/or increments in maternal plasma placental lactogen concentration.

Methods Employed: Twenty helathy female volunteers between the ages of 18 and 30 will be considered for this study. When approval for interruption of pregnancy has been obtained, the patient will be interviewed and if found acceptable will be asked to participate in the study. The responsibility for recommending interruption of pregnancy. for the choice of procedure used for interruption, and for the surgical management is that of physician other than the investigator. After receipt of an informed consent, the patient will be scheduled for admission to the Clinical Investigation Unit of the Johns Hopkins Hospital. The patients' activity is restricted to the ward. Sequential samples of maternal blood, maternal urine, and amniotic fluid are obtained.

Major Findings: It appears that there is a selective response on behalf of the pituitary gland, depending upon the amino acid infused; thus the infusion of arginine in pregnant women, following a fast, in the first 20 weeks of gestation, is not associated with marked rises in growth hormone; whereas prolactin increments are significant. The infusion of alanine, on the other hand, is not associated with any significant increments in prolacting but with marked increases in the release of growth hormone.

The laboratory is currently completing all the radioimmunoassays for growth hormone, insulin, placental lactogen and prolactin as well as the amino acid profiles on aliquots obtained from 80 fasts.

Significance to Bio-Medical Research and to the Institute: The role and import of human placental lactogen (HPL, human chorionic somatomammotropin) and prolactin (HPr) in pregnancy remains undefined. There is preliminary evidence that acute starvation of the human mother, in the first half of pregnancy, is associated with moderate to high rises of maternal plasma HPL concentrations that then return to baseline with feeding. Furthermore, there is also preliminary evidence that maternal plasma HPr concentrations rise and amniotic fluid HPr concentrations fall in pregnancy. The relationship of maternal-fetal nutrition to these changes is not known.

Proposed Course: The contract fiscal year ends April 30, 1973. A renewal is not necessary or planned.

Honors and Awards: None

Publications: None

NICHD Annual Report
July 1, 1972 through June 30, 1973

Gerontology Research Center
Office of the Chief

Research Services

The Technical Development Section has continued its maintenance and development services to the scientific programs of the Center. Several new instruments have been designed and constructed along with day-to-day maintenance services and technical consultations. Advances have been made in the capabilities of the GRC computer system.

A precision muscle suspension system with temperature controlled muscle chambers and specially designed muscle clips and electrodes was designed and constructed for studies on age differences in cardiac performance in the rat.

An automated device for recording of reaction time was developed for the Longitudinal Study. Reliability of the test data will be increased by the automation of both the stimulus presentation and data recording.

The Psychophysiology Section was assisted by the development of a blood flow integrator for use in its studies of long term conditioning of blood pressure and cardiac output in the monkey. A unique feature of this instrument is its capability to eliminate the baseline drift usually encountered in these measurements.

Several instruments were designed and fabricated for the Section on Human Learning and Problem Solving. Visual stimuli are presented with the use of a new control timer, and data gathered and recorded on a new, more reliable printer for the programs concerned with serial and paired-associate learning. A stimulus programmer, utilizing paper tape, automatically sequences visual stimuli for a study on short term retention. An interface with the GRC computer system has been completed which will, with the completion of software, present complex stimuli in a concept-learning experiment.

In the past year there has been a sizable increase in the usage of the GRC computer system. Approximately fifteen different programs regularly use the "background" capabilities of the system to reduce data and perform computations. The Technical Development Section supports these efforts by training programmers, aiding in the debugging of programs, and maintaining the computer system. The capabilities of the system have also been increased. An FM analog tape recorder has been added to allow the reduction of pulmonary and cardiovascular data. The required interface was designed and installed along with general purpose software by the TD Section. An interval timer was constructed and installed in support of the on-line pulmonary testing system being developed for the Longitudinal Study. General purpose software developed by the TD Section include programs which (1) fit

polynomials and non-linear functions to data, (2) allow easy digitization of signals from the analog tape system, (3) simplify the plotting of data, and (4) allow the punching of protocol tapes for laboratory stimulus controllers.

The Photography and Arts Section provided drawings, photographic prints, and projection slides for support to 46 GRC scientists and 5 guest scientists.

There has been a substantial increase in requests for projection slides. In order to meet this increased work load, a new procedure was developed for direct positive slides to eliminate the need for intermediate film negatives.

During the past year, the Animal Resources Facility has provided veterinary services, technical assistance, and animal care for the expanding research program of the GRC. The number of animals cared for has increased significantly this year. The production colony of aging, outbred Wistar rats has been maintained at a level of 10,000 animals. In addition, care has been provided for 9,000 mice, 900 rabbits, 600 rats, 82 dogs, and 10 Rhesus monkeys acquired from outside sources.

This year a total of 1,085 rats have been issued from the production colony to 19 investigators representing every branch of GRC and to five guest scientists from outside institutions.

A small colony of 1,600 aging mice is being maintained for the Laboratory of Cellular and Comparative Physiology. Another colony was set up to breed five strains of mice acquired from the University of Edinburgh. Three strains consist of inbred mice and the other two strains are mutants which are maintained by a backcross with C57B1/6 males. This colony, which now holds approximately 500 mice, is being used in genetic studies.

In collaboration with the Laboratory of Cellular and Comparative Physiology, a detailed survey of pathology present in rats in the production colony has been initiated.

A laminar-air-flow system has been installed in one of the animal holding rooms. This system should be capable of holding up to 1,400 mice while also providing a flexible barrier against environmental stress and contagion. The practicality and effectiveness of this system will be tested during the coming year.

Surgical facilities, equipment, and technical assistance were provided to investigators conducting experiments which required the use of surgical procedures. This year, the technique of implanting an indwelling, electromagnetic, arterial flow sensor around the base of the aorta was applied and developed for use by the Laboratory of Behavioral Sciences. Rhesus monkeys undergoing this procedure have survived for 7 weeks before being sacrificed for pathological examination.

Information Services. GRC internal communication was further improved with the advent of a Center newsletter in January 1973. This

newsletter, developed by the Public Information Officer after consultation with employees and administrative staff, is proving an effective vehicle for informing staff about official GRC operations and general activities of interest to everyone working in the facility.

Media inquiries rose this year to 128 from the 118 reported last year. Stories dealing with the Center or its programs appeared in the New York Times, Saturday Review/The Sciences, the National Observer, the Washington Star-News, Newsweek, the Baltimore Sun, and National History Magazine. Additional contacts were made with Newsday, the New York Daily News, the Wall Street Journal, Time, Fortune, and two German magazines, Der Spiegel and Stern. Medical World News and Chemical and Engineering News also carried articles on the Center.

Electronic media contacting GRC included CBS-TV, BBC-TV, WBAL-TV, and WCAO-Radio (Baltimore), the Voice of America, Radio Free Europe, World Health Organization Radio, Italian RAI-TV, and Ontario Educational TV.

A total of 157 people participated in 42 tours of the facility, including visitors from Australia, Denmark, Germany, and Israel.

The GRC Public Information Officer set up and operated a working Pressroom for the annual meeting of the Gerontological Society at that group's request.

Library. In addition to providing the usual services to GRC investigators, the Library staff continued to search out and index the world literature relating to gerontology and geriatrics. During the year over 3,600 references were identified and classified. The reference lists were published quarterly in the Journal of Gerontology.

Collaborative Guest Scientist Program

During the year, 15 guest scientist programs were supported by the GRC. These programs were staffed by 35 investigators with 30 supporting personnel. Guest scientist programs included studies of cardiac performance in the rat, skeletal muscle physiology, cell biology, gastrointestinal physiology, and endocrinology.

Cardiac Performance in the Rat. Isolated heart muscle (left ventricular trabeculae carneae) from hearts of young and old rats was subjected to anoxia (95% N_2 - 5% CO_2), paired pacing (post extra systolic potentiation), and exposure to norepinephrine at varying concentrations. Contractility of the heart muscle was increased by norepinephrine and paired pacing and decreased by hypoxia. Although there was no significant age difference in the threshold dosage of norepinephrine, at higher dose levels the inotropic response was less in the old than the young. No age differences were found in the response to hypoxia or paired stimulation.

These preliminary results suggest that aging in the rat heart is

associated with a decrement in the magnitude of the specific inotropic response to norepinephrine. The lack of an age related increase in hypoxic sensitivity suggests that there are no major alterations in anaerobic metabolism associated with aging. The decrease with age in the response of cardiac muscle to catecholamines would likely be reflected in decreased "cardiac reserve"--a decreased ability to meet increased stress.

Aging of Skeletal Muscle. Previous studies have indicated that chronically denervated muscles in the rat had a lower collagen content (less total hydroxyproline) than the contra-lateral normal muscle. The current study was designed to isolate age effects, compensation effects, and denervation effects on the collagen metabolism of old and young animals. It was found that the hydroxyproline concentration in normal muscles increased with growth, leveled off at 16-22 months of age, but increased markedly at 28 months. Following denervation there was a reduction in muscle weight. The hydroxyproline content of the muscle increased significantly in both young and old rats, but the increase was slightly greater in the old than in the young. Age of the animal does not seem to influence the effects of denervation significantly.

Tumor Incidence in Senescent Rats. One of our guest scientists is examining the incidence of tumors and other pathology in pituitaries, thyroid glands, and kidneys in young and senescent rats. Preliminary results show a low incidence of thyroid tumors in both young and old rats, but adenomas of the pituitary gland have been found in 75% of the senescent rats (aged 24-26 months). None have been found in 12-18 month old animals.

Cell Biology. One hypothesis of cellular aging and death is that protein synthesis is impaired with advancing age. To test this hypothesis a guest scientist studied the incorporation of ^{14}C leucine or a mixture of ^{14}C labeled amino acids into cold TCA insoluble material by liver microsomal fractions from mature adult (12 month old) and old (20-31 month old) female rats. A decreased amino acid incorporation (ranging from 12 to 43% in individual experiments) into protein occurred in the cell-free system from senescent rat livers. Also, cross-mixing experiments indicated that the cytosol (105,000 g supernatant) from senescent animals inhibited protein synthesis by microsomes from 12 month old rats. The combination of cytosol from 12 month old animals with microsomes from senescent animals functioned like the completely senescent systems. These results support the hypothesis that protein synthesis is impaired in senescent cells. The inhibitory effect of cytosol from aged cells is a new finding of considerable interest.

One of the current theories of aging at the cellular level is that cells can undergo only a finite number of divisions. This phenomenon has been demonstrated by Hayflick in some cells, namely, the WI-38. However, it is not known whether this phenomenon occurs in other types of cells. One of our guest scientists is utilizing regeneration in the earthworm to test this hypothesis. This model was chosen because cells from different segments of the worm have different regenerative potentials. Worms cut at the 90th segment will regenerate 10 segments, while those cut at the 50th segment will regenerate 40-50 segments. The experiments will test in tissue culture

the number of cell divisions that can be carried out by cells removed from different segments of the worm. New techniques have been worked out and, although the initiation of growth is delayed, blastema cells from the earthworm can now be cultured in vivo. A variety of cell types are obtained, so that cloning will be necessary. It has also been found that the brain is not necessary for regeneration in the earthworm.

In view of the Hayflick phenomenon, studies on age-related changes in normal cells in culture can provide in terms of model systems important information about the cellular bases of aging. Using the cell culture technique, it has been found that DNA synthesis is exponentially related to age. Cell age can be correlated with certain ultrastructural changes in cultured cells, such as an increase in secondary lysosomes. Older cells have a reduced capacity to take up exogenous proteins. Some of the age-related changes can be modulated by cortisol and other similar hormones. As in the intact organism, the responsiveness of cultured cells to a hormone signal declines as the cell ages. These cellular processes may provide the basis for understanding the age associated impairments in physiological control mechanisms.

It was shown that when human fetal fibroblasts were transferred from in vivo to in vitro conditions, the cells undergo functional and ultrastructural changes that reflect possibly altered metabolic activities. Cells under in vitro conditions show marked hypertrophy of the rough endoplasmic reticulum, indicative of increased protein synthetic activity. The most striking change consists in a marked enhancement of pinocytosis and stimulation of the lysosomal system, which clearly reflects a change in the nutritional mechanisms in the cultured cells. Macromolecular uptake or bulk transport becomes an important means for internalization of materials from the external environment required for meeting the nutritional requirements under in vitro conditions.

Possible alterations of the adenyl-cyclase, cyclic AMP, phosphodiesterase system during in vitro aging might result in the impairment of key metabolic reactions in the cells mediated by this system, which could be responsible for the deterioration of cell function and loss of reproductive capacity that characterizes aging in cell culture conditions. The WI-38 cell line provides a model system for studying changes in the metabolism of cells as they "age". These changes in metabolism are an expression of control mechanisms. One such control mechanism of system involves cyclic AMP. The synthesis and degradation of this compound have been shown to be important factors in the regulation of cell metabolism. At present, activity levels of adenyl-cyclase and P.D.E. are being determined as a function of passage levels in order to see whether there are changes with cellular "age" (low vs. high passage). If there are changes in the activity of these enzymes, can changes in intracellular cyclic AMP concentrations be correlated with these changes?

Preliminary studies on the activity levels of adenyl-cyclase suggested a marked decrease in enzyme activity in SV-40 transformed fibroblasts when compared to normal cells. In addition, with the more sensitive adenyl-cyclase assay, it has been possible to demonstrate an increase in

adenyl-cyclase activity in older passage cells when compared to the younger passage cells. There does not seem to be any change, however, in the activity of P.D.E. when comparisons are made between young and old cell passages. Further, work recently begun suggests that AMP_c may play an important role in the regulation of growth in WI-38 cells as measured by both absolute increases in cell population and the proportion of nuclei incorporating radio-labeled thymidine.

Results of other experiments comparing uptake and passage level (age) demonstrated marked differences in the absorptive capability of Phase I-II and Phase III cells. Specifically, young (Phase I-II) cells took up about 0.5 nanograms media protein per microgram cell protein during a 4-hour incubation, while old (Phase III) cells pinocytosed 0.3 nanograms media protein per microgram cell protein in the same period ($P < .01$). These patterns of uptake were found to be consistent regardless of the proportion of total protein in the pulsing medium.

Accumulation of lipofuscin "wear and tear" or age "pigment" is one of the most striking cytological changes observed during aging. Male sex accessory organs have been used as a model for investigating in vivo deposition of this pigment during aging. The rationale for using this model is as follows: The cytological mechanisms of formation of autophagic vacuoles after castration and the evolution of these bodies are similar to those observed in normal rats during aging, where many of the lipofuscin granules originate from autophagic vacuoles. In the castrate, the induction of the autophagic process can be controlled, and as it takes place rapidly and in abundance the various stages in the formation and evolution of autophagic vacuoles is readily detected. Furthermore, the fact that autophagia induced by castration can be reversed by androgens, whereas spontaneously developed autophagia during aging is irreversible by any procedure known, offers a unique opportunity for investigating the pathologic factors responsible for the chemical changes in the latter that result in the accumulation and occupancy of large portions of the cytoplasm by metabolically inactive residues.

Studies of the three major components of the male sex accessory tract have shown clearly that lipofuscin originates from both autophagic vacuoles and phagosomes concerned with autodegradation of obsolete cytoplasmic organelles and absorbed exogenous materials respectively. In early stages, these digestive vacuoles contain abundant lysosomal enzymes, but as the intravacuolar digestive process proceeds, enzyme activity decreases and finally disappears. The following course of events can be postulated in relation to subcellular mechanisms involved in the genesis of lipofuscin from autophagic vacuoles and phagosomes. Obsolete or denatured cell organelles and pinocytosed materials within digestive vacuoles undergo digestion of the protein component of lipoprotein complexes that enter into the formation of cell organelle membranes. The lipid part, probably due to poor lipolytic activity of lysosomal enzymes, remains undigested and precipitates in the form of membranous arrays--so-called myelin figures. In later stages these membranous arrays evolve into a homogeneous lipid-like material, which presumably represents lipofuscin or some other inert residue that cannot be further degraded by the enzymatic machinery of the cell.

Since plasma membranes are involved in the cellular control of transport and metabolism, they represent potential sites for age changes. A pilot study of the protein composition of plasma membranes from rat kidney was carried out. Membranes were separated by sucrose gradient techniques. The membranes were then solubilized and their protein components were separated by poly acrilamide gel electrophoresis. Animals from three age groups (6-8 mo., 12-14 mo., and over 24 mo.) were studied. Preliminary results show two small peaks in the preparations from old kidneys which are absent in the young. Work is continuing to isolate and characterize the atypical proteins which appear in plasma membranes from senescent rats.

Physiology of the Gastrointestinal Tract. A number of scientists have carried out studies using the gastrointestinal tract as a model system. These studies range from investigations of cellular responses to toxins to investigations of motility and absorption from the intestine.

Using micropuncture of individual cells and recording voltage and resistance across the cell surface, it was found that theophylline causes a depolarization of inter-villus cells of the intestine and an increase in resistance across villus cells. These findings are consistent with the known effects of theophylline on electrolyte transport, that is, a stimulation of chloride secretion and inhibition of sodium absorption. It is suggested that the inter-villus areas principally contribute to fluid excretion. This base-line information is necessary for projected studies of the mode of action of the effects of enterotoxins on the gut.

The mechanisms of interaction of enterotoxins with cells is being studied in order to understand how toxins bind to cells and influence cellular metabolism. The goal is to discover ways to block the action of enterotoxins and to identify the relationships of toxin effects to those of physiologic stimuli.

Epididymal fat pads from young and old rats were incubated with collagenase and a suspension of isolated cells was used for the experiments. It was found that monosialoganglioside, which specifically binds cholera toxin, will enhance responsiveness of fat cells to toxin and raise the responsiveness of cells from older rats to equal those of young rats. Cholera toxin exerts its effect at a site not blocked by A or B adrenergic blocking agents, although there is a slight effect by A blockers such as phentolamine or dibenzyline on response to toxin. Adenyl cyclase is stimulated by both E. coli and V. cholerae enterotoxins. Prior exposure to these toxins enhanced responsiveness of membranes to subsequent challenge by epinephrine.

Cellular responsiveness to enterotoxins in the case of rat epididymal fat cells is clearly age dependent. Discovery that ganglioside binds cholera toxin and enhances cell response to toxin if cells are pre-incubated with this substance allows experiments in which the ability to restore cells from older rats to responses characteristic of younger rats opens the door to specific experiments which could relate the numbers of a specific receptor to aging. Enhanced response by cell membranes to hormones after exposure to toxins could be an important parameter to study in cells from animals of different ages.

Enterotoxins were also prepared from culture filtrates of E. coli sterilized by filtration and concentrated by lyophylization. Biological activity of the enterotoxins was assayed in the infant and adult rabbit ileal loop model. Antitoxin assays were performed by measuring neutralization of enterotoxin in the adult rabbit ileal loop. Immunization studies were done on adult rabbits using antigen mixed in incomplete Freund's adjuvant as the primary immunization method. Challenges will be made of the ileal loops of the immunized rabbits.

Major findings were: Enterotoxins from E. coli isolated from children with diarrhea from Arizona and Alexandria, Egypt, have been identified. Neutralization of six different E. coli enterotoxin preparations (each from a different E. coli serotype) by the standard E. coli antiserum was demonstrated. These E. coli strains were isolated from India and the United States. Immunological similarities between enterotoxins of E. coli and V. cholerae were demonstrated by neutralization tests. Preliminary results suggest that children infected with enterotoxigenic E. coli strains develop antitoxin titers during convalescence. As of this report, the experiments involving challenge of immunized rabbits are still in progress.

Diarrheal disease due to E. coli is primarily a childhood disease. Why the disease is seen less frequently in adults is not known, but may be related to immunologic processes. The finding of a common enterotoxin antigen suggests that immunization could be useful in control of the disease.

Studies on the effects of enterotoxins and pharmacologic agents on gut secretion were carried out in dogs by measuring net secretion, mucosal adenyl catalase activity, time course of toxin effect and mucosal histology. The effect of desoxycorticosterone (DOCA) on small bowel electrolyte transport was studied in dogs with Thiry-Vella loops of duodenum or ileum. Electrolyte content and volume of secretions induced by cholera toxin were studied before and after 3 or 6 days of DOCA administration.

There was no structural abnormality in mucosa during toxin-induced secretion. There was no increase in clearance of protein from serum to gut lumen. The E. coli toxin appears to be deactivated or consumed as a result of exposure to the gut, thus a sustained secretory response requires continuous application of toxin. Control "toxin" from a nontoxigenic strain of E. coli produced none of these effects.

The time-courses of heat-stable (ST) and heat-labile (LT) E. coli toxins were different in rabbit small bowel. ST effect was immediate in onset and of brief duration, the maximum cumulative secretory response being at 4-6 hours. By 18 hours ST effect was usually gone. LT effect was slightly slower in onset and its duration depended upon total dose of toxin. At low doses it peaked at 10 hours, at higher doses it peaked beyond 18 hours. The time of peak effect of cholera toxin was not related to dose, being beyond 18 hours even at submaximal doses. LT was neutralized by antibody to whole E. coli toxin but ST was not. The optimum time to assay ST was 6 hours, while that for LT was 18 hours.

DOCA has the previously unrecognized effect of stimulating potassium

excretion by the distal ileum, but not the duodenum, during gut secretion induced by cholera toxin. It had no effect upon secretory volume.

Acute expansion of extracellular fluid volume in dogs caused marked secretion by the small bowel. This secretion was not associated with alterations in mucosal adenyl cyclase activity.

Studies on the binding of E. coli and cholera enterotoxin to gut mucosa indicated that ganglioside binds heat-labile but not heat-stable E. coli enterotoxin. This is in contrast to the previous report that ganglioside did not bind E. coli toxin. The binding of E. coli LT by ganglioside appears much less efficient than that of cholera toxin. This observation is being pursued as a possible means of purifying E. coli toxin. Affinity chromatographic techniques are being used to attempt to bind LT to ganglioside and then elute it from the complex of ganglioside and agarose.

In studies elsewhere further evidence is accumulating that ganglioside (specifically the sialidase resistant monosialoganglioside) is the membrane component of mammalian cells which binds and is the receptor for cholera toxin.

Antitoxic immunity to cholera has been further investigated to clarify the roles of serum or locally produced antitoxin in protection against cholera. Antitoxin titers have been measured in serum and jejunal washings after local immunization or active or passive parenteral immunization.

It was found that both serum and locally derived antibody can protect against toxin challenge. Serum antibody is largely lgG whereas local antibody is IgG and lgA. Passive immunization by intravenous serum administration yielded measurable intraluminal antibody and protected against toxin challenge. Intraluminal toxoid administration was not effective in boosting serum antitoxin titers.

Dogs immunized parenterally or with parenteral plus oral glutaraldehyde toxoid were protected for at least 1 month to vibrio challenge. Serum titers were low to toxoid alone but were markedly improved by use of an aluminum adjuvant.

Cholera antitoxin titered in rabbit gut segments gave results similar to those in rabbit skin. However, at low levels of activity the rabbit gut system appeared more sensitive than rabbit skin.

Serum vibriocidal antibody induced by a subcellular vibrio vaccine was unprotective despite high serum vibriocidal antibody titers. This indicates that this antibody is not responsible for protection induced by whole cell vaccines.

Serum titers of vibrio motility inhibiting antibody increased after parenteral immunization with whole cell vaccine. These titers appeared independent of agglutinating antibody titer. They have not yet been measured in gut washings.

The everted gut technique has been used to study age differences in

intestinal absorption of folate in the rat. No significant differences were found in the absorption of folate in 6, 12, and 24 month old animals.

Motility of the Gastrointestinal Tract. Esophageal Studies: Biofeedback control of lower esophageal sphincter contraction was achieved using tandem open tipped catheters to measure pressures from the esophagus, lower esophageal sphincter and stomach in normals and patients with esophageal reflux. Instantaneous biofeedback was provided to the subjects by means of a meter. Both normals and patients with reflux esophagitis were able to increase lower esophageal sphincter pressures without corresponding changes in intraesophageal, intragastric pressures or in respiration. This skill persisted after biofeedback was discontinued. These studies demonstrate the ability to condition autonomic gastrointestinal activity by biofeedback techniques and open the possibility of operant control of disordered gastrointestinal functions, including reflux esophagitis, which occurs more commonly with increasing age.

Anorectal: Adult patients with severe intractable fecal incontinence (continuous daily soiling) were tested to evaluate manometric and clinical effects of biofeedback training in the control of anal sphincter responses. Pressures were measured by triple balloon system from the rectum, internal and external anal sphincters and sphincteric reflexes were initiated by transient distension of the rectal balloon (simulating arrival of stool in the rectum). All incontinent patients demonstrated normal internal sphincter reflexes but absent or markedly impaired external sphincter responses emphasizing the role of the external sphincter in maintenance of continence. Instantaneous visual feedback was provided by permitting patients to observe the direct writing records. Patients were taught to contract the external sphincter in synchrony with internal sphincter relaxation. The success of this technique in achieving fecal continence even after visual feedback was discontinued indicates the importance of synchronized sphincter contraction for maintenance of continence and the potential value of these techniques as a simple means of treating fecal continence, a problem which increases in incidence in an aging population.

Colon Motility and Compliance. Recordings of rectal compliance and colonic motility from the rectosigmoid, rectum, and internal anal sphincter in normals and in patients with irritable bowel syndrome demonstrated two types of colonic responses to rectosigmoid distension in normals and an additional "spastic" contraction in patients with irritable bowel syndrome. Active colonic contractions were usually associated with sensation of gas or an urge to defecate, depending on the location of the spasm. The aggravation of these contractions by feeding correlates with clinical observation that symptoms of irritable bowel syndrome are aggravated by food intake. These studies are being applied to patients with irritable bowel syndrome along an aging continuum, in light of recent information that irritable bowel syndrome may be a precursor of diverticular disease and that diverticular disease increases appreciably with increasing age. Biofeedback techniques will be employed to attempt inhibition of exaggerated spastic responses as a means of treating this disorder.

Osteoporosis. Although osteoporosis and loss of calcium from bone

occurs with advancing age, precise measurements of bone density in intact human subjects has not been possible. Recent technical advances (photon absorbtiometry) now permit a precise assessment (±4%) of bone mineral content in intact humans. A collaborative project to follow changes in bone mineral content has been initiated, using subjects from the Baltimore Longitudinal Study. A preliminary analysis of observations on 107 subjects (age range 25-90) failed to demonstrate a significant correlation between bone mineral content and age.

The double-blind clinical trial of salmon calcitonin in the treatment of symptomatic osteoporosis has been suspended because of recall of the therapeutic agent by the manufacturer because of its previously unappreciated short shelf life. The design of the trials involved 90 days treatment which could not be carried out with a single preparation of calcitonin.

Thyroid Studies. The hypothesis that one of the mechanisms of aging is a reduction in the effectiveness of physiological controls is also under investigation. Since hormones play an important regulatory role, their action at the cellular level is being studied by one of our guest scientists. It has been found that there are specific intracellular proteins which bind thyroid hormones. Physical properties of these proteins have been characterized, including mobilities in several electrophoretic systems, molecular weight, isosonic point, affinity and behavior in various chromatographic systems. This S protein from dog liver and kidney has been purified 150-fold. The cellular cytosol binding proteins are distinct from serum carriers of thyroxine and tri-iodothyronine and are presumed to modulate intracellular actions of these hormones. Current work involves quantitation of these proteins and studies of cytosol protein-nucleic interaction.

Amyloidosis and Aging. Senile amyloidosis is a significant disease of the elderly. Cardiovascular amyloidosis in particular appears to be associated with a significant morbidity. At present there are no accepted or proven forms of therapy for amyloidosis, and studies directed at possible mechanisms of amyloid resorption represent important steps toward this end.

Recent studies suggest that amyloid fibrils may be phagocytosed under certain conditions. In tissue cultures, for instance, amyloid fibrils may be phagocytosed by blood macrophages provided antiserum to alkali-denatured amyloid fibrils is added. Recent observations suggest that, in the rabbit, spontaneous amyloid resorption in the spleen occurs within a defined time period following amyloid induction.

The introduction of purified rabbit amyloid fibrils into the subcutaneous tissues of BDF inbred mice resulted in resorption of the fibrils within 5-9 days. This was preceded by an accute inflammatory response, but during resorption the cellular reaction was predominantly macrophagic. Acid phosphatase preparations showed that macrophages nearest the residual amyloid deposits were particularly rich in lysosomes and light microscopy revealed intracytoplasmic inclusions of amyloid. Electron microscopic studies are currently underway.

Intramural Research

The Laboratory of Molecular Aging. Research projects, utilizing a variety of model systems, have examined questions related to regulatory processes in the transmission of genetic information, mechanisms of control of enzymatic activity and cellular metabolism, and biochemical changes during aging that alter the responses and functions of subcellular organelles and cells.

An extensive study of infection of human cells (WI-38) in culture by vesicular stomatitis virus and its prevention indicates: (1) Cellular viral defense, i.e., the induction of interferon, is functional throughout the life span of the cells up to the moribund state, but the amount of poly IC required for induction increases with age. (2) In senescent cells the polynucleotide inducer of interferon produces the antiviral state faster than treatment with interferon itself, suggesting either another antiviral protection mechanism in senescent cells or a change in the relative rates of uptake of poly IC and interferon in the senescent cell.

Synthetic analogs of nucleic acids, representing modifications of the structure of native genetic material, have been shown to interfere with specific biological processes and, therefore, can be used to study biochemical mechanisms. Thus, polyvinyl uracil and polyvinyl adenine both inhibit murine leukemia virus infection. The inhibition of the leukemia virus appears to be related to the inhibition of a RNA-dependent DNA polymerase.

Complexes of platinum have been used to explore the mechanism by which the replication of DNA and synthesis of RNA are impeded by metal ions. It is known that the cis-form of a platinum complex inhibits DNA replication, cell division, and is an effective antitumor agent, whereas the trans-form of the complex is not active in these respects. It has now been found that the cis-isomer, but not the trans-isomer, forms complexes with DNA. The Pt-containing complex inhibits transcription in an in vitro system, and this inhibition is on the polymerization process, without affecting the initiation process. A technique has been developed to replace the zinc, the natural metal in RNA polymerase, with several metals including copper and to retain enzymatic activity. The substitution of copper, allowing electron spin resonance studies, may provide a valuable tool in elucidating the molecular action of the enzyme.

Studies on the mechanism of active transport of sugar in the kidney have continued to make significant progress and several underlying principles have been established. Glucose transport by isolated renal brush border membranes is comprised of at least two processes, a high affinity binding system and a carrier-mediated transport system. The presence of a stereo-specific high affinity binding system has been unequivocally demonstrated by equilibrium dialysis. Kinetic studies of the binding have characterized its time course, reversibility, saturability, susceptibility to the presence of phlorizin, and have determined the effects of Na^+ as well as Ca^{2+} and Mg^{2+}. A particulate fraction derived from detergent-extracted brush border membranes has been prepared. This preparation has the same affinity and capacity as intact membranes but has lost about 90% of its

protein content and shows no evidence of vesicular structure. Evidence for a glucose-bound protein has been obtained. The mechanism of uptake of high concentrations of glucose by the intact brush border membranes shows both influx and efflux components and is consistent with a hypothesis for a carrier-mediated transport into and out of vesicles. Prior loading experiments demonstrate the phenomenon of counter-transport or facilitated exchange diffusion. Although Na^+, per se, is not required for uptake of glucose by the brush borders, the rate of glucose transport by the membrane is greatly enhanced by a Na^+ gradient from the outside to the inside of the membrane.

Extensive studies on the control of pyruvate oxidation in mitochondria from blowfly flight muscle, and confirmed with mitochondria from rabbit heart muscle, have led to a novel hypothesis to explain the regulation of Krebs cycle activity. Both ADP and inorganic phosphate are required for the uncoupled (FCCP) oxidation of pyruvate as well as for the coupled oxidation. The FCCP-stimulated oxidation is markedly inhibited by physiological levels of Ca^{2+}. The apparent $K_I = 10\mu M$. The electron transport chain of the mitochondria is fully functional in the presence of Ca^{2+} since the oxidation of α-glycerolphosphate is not inhibited, indicating control at the dehydrogenase level. Kinetic studies show that the Ca^{2+} inhibition is uncompetitive with respect to ADP and FCCP, but complex, and noncompetitive with respect to pyruvate. The results of these and other studies suggest that pyruvate oxidation and Krebs cycle activity is limited by NAD-dependent isocitrate dehydrogenase activity and Ca^{2+} plays a role in its regulation. An additional mechanism for the control of pyruvate metabolism has been demonstrated in mitochondria for the first time. In analogy to glycogen phosphorylase, this involves the phosphorylation and dephosphorylation of the pyruvate dehydrogenase complex by a kinase and phosphatase, respectively. In contrast, however, with pyruvate dehydrogenase, the phosphorylated form is inactive whereas the dephosphorylated form is active.

A correspondence between the decline in the ability of aged blowflies to fly and in the maximally stimulated respiratory rate (State 3) and respiratory control ratio of flight muscle mitochondria was reported previously. The biochemical mechanism and locus of this decrement with senescence has been investigated further by examining the bioenertetic properties of mitochondria from mature and aged flies for rates of oxidation during coupled oxidative phosphorylation, activities of the dehydrogenases, concentrations of components of the electron transport chain, phosphorylation of ADP, and the coupling mechanism itself. Evidence, to date, indicates that the specific activities of α-glycerolphosphate and pyruvate dehydrogenases are unaltered by age. Similarly, the concentrations of cytochromes a, a_3, b, and $c+c_1$ are the same for both age groups. ATP synthetase, measured as ATPase, is unchanged with senescence. In contrast, the uncoupled oxidation of substrates is significantly decreased with aging, showing the 30-40% decrement between mature (7-10 days) and aged (31-33 days) as seen previously for the maximally stimulated coupled rate of respiration. These observations narrow the possible loci of the defect in aging to the bioenergetic system between the primary dehydrogenase and the FCCP-sensitive uncoupling reaction.

Hormonally (parathyroid, synthetic 1-34, and catecholamine)

stimulated adenyl cyclase has been demonstrated in renal cortical membranes. Luminal brush border membranes bind c-AMP, as determined by equilibrium dialysis and Millipore filtration experiments. Kinetic parameters of the binding have been examined. It is anticipated that the binding of c-AMP may initiate kinase activation and potentially exert a controlling role in renal tubular function.

Laboratory of Cellular and Comparative Physiology. FY'73 has been highlighted by a major change in the emphasis of program goals and in the accompanying research activities to achieve these goals.

The current goals are three-fold: (a) To conduct studies on the nature of age-related deteriorating functional changes of cells and tissue systems whose growth and differentiation events are reasonably well understood, (b) to determine the underlying mechanisms of these changes, and (c) to develop methods for early detection of the deterioration and, hopefully, methods to control or reverse the deterioration.

To implement these goals emphasis will be on the use of cells and tissues of mammalian species, including man, in in vitro and in vivo cultures. Moreover only a few closely related model systems will be analyzed, but they will be studied systematically.

The major thrust in research activity has been in immunology and aging, with the creation of the Immunology Section, for the following reasons. (1) Associated with, or as a consequence of immunosenescence, the incidence of immunodeficiency diseases including autoimmunity, cancer and infection increases with age. (2) Cellular, molecular and genetic knowledge of the ontogeny, phylogeny and differentiation process involved with the immune system is well understood, probably more so than any other functional system. (3) The system is amenable to elegant cellular and molecular analyses and, therefore, offers great promise for successful manipulation of the system.

During the past year, members of the Immunology Section expanded their effort in the creation of the laboratories and initiation of research projects. Hence fruition of their current research task should become apparent at the earliest during the next fiscal year. Nevertheless, basic findings, some of which were established just prior to their arrival at the GRC, are as follows.

(1) Of the three cell types (thymus-derived T cells, bone marrow-derived B cells, and accessory A cells) involved in the initiation of a humoral immune response, the T and B cells are altered in immunologically deficient old mice, but A cells appear normal.

(2) The proliferative capacity of T and B cells is decreased as much as 10-fold with age. This is a very critical function because the number of functional effector cells generated in an immune response is dependent upon the ability of these cells to divide efficiently.

(3) Limiting dilution, reconstitution, and young cell-old cell mixture

analyses offer indirect evidence that either the relative number or the efficiency of regulator cells that suppress an immune response increase with age. For example, it was found that the humoral immune activity of a mixture of spleen cells from young and old mice was lower than the sum of individual responses, indicating that there are cells in old mice that can inhibit young immunocompetent cells from responding to antigenic stimulation.

(4) One of the deficiencies of radiation-induced allogeneic bone marrow chimeras was shown to reside in the thymus gland, the seat of immunodeficiency diseases in most cases. Previously it was shown that these chimeras are suitable model animals to study immunosenescence.

(5) Limiting numbers of lymphoid cells undergo maximum antibody response in vitro only when they are cultured under optimum cell concentration, a variable which was not taken into consideration previously. This information offers us the option of assessing quantitatively the activity of limiting numbers of lymphoid cells and, hopefully, one or two clones of competent cells.

(6) Mice with an exceptionally long life span show a decline with age in cell-mediated immune activity and resistance to allogeneic tumor cells. Previously the decline in activity was observed in mice with short life spans. Therefore critics have argued that life-shortening diseases imposing on the immune system were responsible for the decline in cell-mediated immunity. These results should minimize such criticisms.

Based on these findings efforts are focused currently on the cellular and molecular definitions of immunosenescence with emphasis on (a) isolation and characterization of promotor and suppressor cells, (b) ability of stem cells to generate thymus-derived T and bone marrow-derived B cells, (c) life spans of memory cells, (d) mitogen receptor sites and the cyclic AMP system of proliferating T and B precursor cells, and (e) characterization and repair of induced and naturally occurring immunodeficiency states.

Research has also been initiated on a small scale in biomembrane physiology, with emphasis on the effect of age on hormone bindings, for two reasons: (a) It will complement ongoing molecular studies in immunology and (b) the time is "ripe" to initiate cellular and molecular studies focused on the nature of inefficient response to hormones and drugs by the aged. Preliminary results showed that adipose tissue, skeletal muscle, brain and prostate gland of aging rats exhibit a progressive decline in steroid hormone binding due, in part, to a reduction in the number of macromolecular binding sites per unit tissue mass.

Another area of research that can contribute to the program goals of LCCP is mammalian genetics. An understanding of the genetic mechanisms of age-related functional deterioration at the molecular level is obligatory. To this end, research is being promoted to understand, at the cellular level, human and animal disorders manifesting altered development and aging. Currently a laboratory is being created to carry out two studies: (a) The role of chromosomal aneusomy in the impaired proliferation of fibroblasts and immunocompetent cells in relation to aging and (b) the influence of gene

dosage and maternal environment on the life span of embryos and individuals carrying lethal genes. The long-term goal of these studies is to "sort out" and elucidate those genetic alterations which can impair the proliferative capacity of somatic cells. Information derived from this program should have a tremendous impact on cellular biology of aging, for impairment with age of the proliferative capacity is characteristic of certain somatic cells.

Two sections in existence prior to FY'73 are the Nutrition and Morphology Sections. The former is concerned with the influence of nutrition and antimetabolites on the life span and various tissue enzyme activities in rats. The latter is concerned with structural and cytochemical changes associated with development and aging of myocardial and skeletogenic cells and with the growth patterns of coelenterates as influenced by temperature and endogenous rhythmic changes.

During the past year, the Nutrition Section completed preliminary studies in two areas: (a) The effect of cycloheximide on the rate of development of chick embryos and (b) the effect of protein undernourishment on the life span of middle-aged rats. In the former study it was found that nontoxic levels of cycloheximide, an inhibitor of protein synthesis, when given to 1 day old chick embryos delay their rate of development at least until day 17, as judged by morphological parameters, heart beat, and DNA content. However, the activities of lysosomal and mitochondrial enzymes were not affected.

The latter study revealed that reduction in the dietary protein intake in 16 month old rats from the standard 24% to 8 or 4% had no influence on their subsequent mean life expectancy. However, rats restricted to 12% casein diet lived on the average of two months longer. The life promoting effect of 12% protein intake was manifested during the first 3-4 months following dietary restraint. In view of the profound implication this observation offers, a more systematic study is being initiated.

Ultrastructural cytochemical studies by the Morphology Section relating lysosomal activities and aging processes in the left ventricular cells of aging rats revealed that in mural myocardial cells there are (a) two different pathways by which primary lysosomes are formed and (b) two different mechanisms of lysosomal degradation of mitochondria. The two pathways of synthesis, transport and packaging of primary lysosomes are the nuclear pole zone route and the peripheral route. The latter, which bypasses the Golgi, provides the cells a more direct route to sites of action of the lysosomes. Lysosomal degradation of mitochondria occurs by the more common matrix densification process and the less common matrix clarification process. Studies on papillary myocardial cells of 24 month old rats showed that there are two types of changes: (a) Cells that appear grossly normal in structural appearance but showing marked intracellular changes in comparison to those of 6 and 12 month old rats, and (b) cells that appear grossly abnormal in structural appearance. Ultrastructural definition of age related deterioration of heart muscle cells should provide us a better insight how to resolve whether the changes observed are the cause or consequence of senescence of the organ.

Studies on endogenous circannual rhythms in growth, development and life spans in marine coelenterates revealed that they can persist for over 3 years in the absence of periodic signals from the environment. The period for the cycles at three ambient temperatures, 10°, 17°, and 24° C, was about one year. The life span of hydranths during prolific growth of the colony was considerably longer than during repressed growth. These observations challenge the generally accepted concept that there is a specific age at which death is characteristic for a species.

Clinical Physiology Branch. Research in the Clinical Physiology Branch continues to be geared toward a physiological and biochemical dissection of mechanisms underlying both adaptive and the deteriorative changes of aging--and, when possible, to accomplish this goal in man.

The invaluable resource for our study of human aging continues to be the Longitudinal Aging Study. This year has been one of continued development and refinement of unique techniques for longitudinal data analysis.

To quantitate age changes in the lung, the forced expiratory volume in one second (FEV 1.0) has been measured. The mean rate of aging within individual subjects can be defined in young and middle-aged adults with less than 25% error and in old subjects with less than 10% error.

Blood pressure increases with age show important trends not previously realized. Blood pressure has been recorded under basal conditions (during basal metabolic rate measurements) and under "casual" conditions, that is, as recorded by a physician during a physical examination. The variance associated with the usual casual conditions of blood pressure recording makes it impossible to detect age changes in systolic pressure of individual subjects with adequate accuracy. On the other hand, measurements of systolic pressure made under basal conditions shows progressively greater rates of increase with advancing age and these age changes within an individual can be defined with statistical confidence.

Serum cholesterol and triglyceride levels show strikingly different aging trends. Statistically significant increases occurred in cholesterol (errors of less than 25%) in young and middle-aged adults, but leveled off after the age of 60-65 years. Triglyceride levels were constant in young adults and fell significantly (errors again less than 25%) in middle-aged and older men. Identification of the reasons underlying the differences in these changes await multivariate analysis of dietary, activity, and other metabolic variables in the coming year.

The versatility of the new analytical technique for determining the design of longitudinal studies is best seen when it is used predictively, i.e., for future planning. It can be shown that most of the physiological variables of interest to gerontologists will require 15 to 30 years of study in order to achieve reasonably accurate estimates of rates of aging in individual subjects across the adult age span. This statistical approach in combination with the basic data provided by the Baltimore Longitudinal Study is an achievement which clearly has far-reaching implications for the future.

The difficult problem of separating age changes from disease changes

has not been dealt with adequately either theoretically or practically by other ongoing studies of human aging. We have devoted much effort the past year to the development of techniques to permit this separation. An automated chart review was the initial step which converted detailed data from medical histories, physical examinations, and laboratory data to an efficiently arranged computerized summary. Comments written by the examining physician are also included in the computerized summary. With these data now available on over 4,000 subject-visits, detailed criteria has been developed for the classification and identification of those disease states which could alter physiological functions independent of age. Detailed classification has been accomplished for pulmonary diseases, renal diseases, coronary heart disease, and cerebrovascular disease. These classifications should provide the standards which other longitudinal studies of human aging will be able to use.

New studies have been introduced into the Longitudinal Study this year. Cutaneous malignancies are a serious problem, especially in older subjects. Studies of chronic actinic damage as a precursor of these malignancies have assumed that the damaging radiation spectrum is 290-320 nm and that longer wavelength ultraviolet radiation (320-400) causes "tanning" and may even protect against sun damage to the skin. A comprehensive study on the effect of age on sensitivity to UV light of different wavelengths and on the time course of the radiation effects is therefore underway. Results to date show that the longer UV wavelengths actually have an augmentative effect, not a protective effect.

Observations on additional genetic markers have been introduced into the longitudinal study in order to assess further the role of genetics in aging. Measurements of haptoglobin, ceruloplasmin, and transferrin in plasma and dermatoglyphic analyses are now being made on all subjects in the longitudinal study.

An interesting analysis has been made of various estimates in use for the determination of body fatness. Not only heights and weights, but also the detailed Behnke Index (which includes abdominal girth), and skin-fold thicknesses have been determined. Many of the subjects in the longitudinal study underwent both gains and losses in body weight during the period of observations. Since the changes in body weight were due almost entirely to spontaneous dietary decisions on the part of the subject, they largely reflect changes in fatness rather than changes in lean body mass. Changes in the individual indices of body fatness were correlated with changes in body weight. It was found that changes in skin folds correlated with loss or gain of body weight with an r of only 0.4; the complex Behnke Index correlated better, r values of 0.64-0.78 in subjects of different ages, while the best indicator of change in fatness was the simple measurement of abdominal girth which correlated between 0.64 and 0.90 in subjects of different ages. It appears that abdominal girth is a distinctly better measurement of fatness than is skinfold thickness.

Study of the chemistry of human renin, a kidney enzyme involved in the pathogenesis of hypertension, has progressed through the use of a new labeled polymeric substrate assay previously developed in the Section on

Endocrinology. This enzyme is inhibited by a variety of proteins and peptides. Studies of the mechanism of this inhibition with a model protein, hemoglobin, with hemoglobin chains, and with chemical and enzymatic cleavage fragments of hemoglobin have shown that large peptides are the most potent inhibitors. The leucine-leucine bond, which is necessary for renin action on its substrates, is not necessary for inhibition by hemoglobin fragments. A number of small synthetic peptides are also potent inhibitors of renin. Renin preparations, free of peptidase activity, have now been shown to contain protease activity against hemoglobin. A new concept of renin emerges in which the previous unique characteristics of the enzyme are succeeded by one in which renin shares many of the characteristics of only relatively specific acid proteases of wide biologic distribution. The techniques and concepts recently developed should permit the development of renin inhibitors of potential clinical usefulness.

The possible involvement of the adenyl cyclase ("second messenger") system in senescence is being explored. This membrane-bound enzyme is activated as the initial step in the action of many hormones. As a result, cyclic adenosine monophosphate (cyclic AMP) is formed; a variety of metabolic processes are then initiated by this compound. Although hints of involvement of this system in certain age-related alterations of hormone action can be extracted from the literature, no actual studies of the enzyme in senescence have been reported to date. Our preliminary data now indicate that, in rat liver, senescence is associated with a marked increase of activation of adenyl cyclase by epinephrine. Although non-specific activation with fluoride is also somewhat enhanced, activation by another hormone, glucagon, is unaffected. These observations indicate the existence of hormone-specific effects of age on membrane structure and function. Moreover, they suggest that altered cyclase responses may provide a molecular basis for the alterated sensitivity to hormones which has been seen with aging. This line of investigation is being extended to include a variety of hormone-activated cyclase responses in a number of tissues.

The nature of the age decrements in myocardial performance has been explored by studies using isolated trabeculae carneae of young, middle-age, and old rats. In these preparations, single and paired stimulation has been given under conditions of exposure to graded levels of norepinephrine and to hypoxia. Biophysical muscle responses have been quantitated. The oldest animals show a reduction in developed tension and in the maximal rate of tension development in response to catecholamines. No age differences occurred in the paired-stimulation and hypoxia studies. The successful development of this experimental procedure and the demonstration of age differences in response to catecholamines in these heart muscle preparations will lead to further pharmacologic studies on the role of the sympathetic nervous system in determining age differences in response of the heart to stress.

Studies have also been carried out on the intact dog heart to test the hypothesis that there is a damaged but salvageable area of heart muscle after coronary artery ligation. The experimental variables in this complex, carefully controlled preparation are (1) myocardial damage as quantitated by ST segment surface mapping, (2) intramyocardial oxygenation using a

polarographic method, (3) coronary artery perfusion pressure. Results show a significant correlation between the ST segment map and myocardial oxygen tension on a minute-to-minute basis in each dog. Furthermore, increasing the coronary perfusion pressure significantly improved oxygenation and decreased the area of ST segment abnormality. There is thus now experimental evidence (1) to support the validity of using ST segment changes as quantitative indices of the ischemic zone and (2) to support the rationale of elevating coronary perfusion pressure to reduce infarct size.

Studies carried out in collaboration with Baltimore City Hospitals and Johns Hopkins University scientists have increased our understanding of myeloma kidney. Thirty-six patients have now been studied prospectively. Changes in urine concentrating and acidifying ability preceded GFR changes. PAH clearance decreased out of proportion to GFR decreases. Only patients with free Bence-Jones protein demonstrated impaired tubular and glomerular function. Renal biopsy showed that changes in renal function correlated closely with tubular atrophy and degeneration, but not with tubular casts. It appears then that Bence-Jones protein exerts a direct toxic tubular effect and that glomerular dysfunction occurs only after severe tubular damage has occurred.

The previously developed model for insulin and glucose metabolism in man has been used to compute the changes in glucose content of two of the glucose compartments when changes in glucose concentration are induced in the third compartment, viz., the blood volume. This computation is critical, especially when large changes in blood glucose concentration occur rapidly. Since one of the experimental variables computed in these studies is glucose utilization by the body, the glucose which has been infused but which has gone only into the extracellular glucose spaces must be subtracted from the total glucose infusion rate. This computation could not be made with any degree of accuracy until a successful glucose model was developed.

The complex biphasic response of insulin secretion in response to hyperglycemia has now also been treated mathematically by the development of a mathematical model of the pancreas. The pancreas model behaves "correctly" in terms of its release of insulin in response to hyperglycemia and it can also reproduce the different insulin responses in young and old subjects. Factors influencing insulin secretion other than glucose concentration and age can also be accounted for by the model. The next goal is the incorporation of the pancreas model into the previously developed glucose and insulin models of the body to close the loop of these interrelated variables.

The glucose-clamp technique (both the hyperglycemic clamp to study beta cell sensitivity and the insulin clamp to study sensitivity to insulin) has been used in a prospective study of the mechanisms underlying the glucose intolerance of uremia. The differentiation of uremic glucose intolerance from diabetic renal disease with uremia is important in terms of decisions concerning acceptance of patients into dialysis and transplantation programs; diabetics with uremia have such a poor prognosis that they have been given very low priority in these programs. Eight uremic patients have been studied. Seven of them showed resistance to insulin, a mechanism for glucose intolerance which is clearly different from true diabetes mellitus. Four

subjects have been studied both before and after chronic hemodialysis. All four showed marked improvement in glucose tolerance as their uremia was ameliorated. The improvement in tolerance could be accounted for by an increase in sensitivity to insulin in three patients and by an increased secretion of insulin in the fourth. Therefore, while both factors may play a role in uremic glucose intolerance, insensitivity to insulin appears to be the more important. Glucose intolerance in uremics thus should not per se make subjects ineligible for definitive therapeutic programs since it frequently is not indicative of true diabetes mellitus.

Laboratory of Behavioral Sciences. During the year the animal behavior program was transferred from the Psychophysiology Section to the Section on Human Learning and Problem Solving, and the name of the latter was changed to the Section on Learning and Problem Solving. The goal of this reorganization was to facilitate the scientific integration of these two programs, and this interaction has already begun to occur.

Previous studies of logical reasoning in the Baltimore Longitudinal Study Program had shown that men over age 60 made significantly more errors than younger men in reaching solutions to complex logical problems when the task required them to integrate the problem solution with the task analysis. The errors were of two kinds: (a) requests for information which was inferable from existing information; (b) redundant requests for information already given. In an analysis designed to separate analytic behavior from synthetic behavior, it was observed that the tendency of the older men to make redundant requests was greatly reduced. This finding indicates that the older men are more likely to make premature attempts to synthesize information than are the younger men, and that this tendency results in repeating solution attempts which have failed.

Problem solving behavior is also being investigated in rats. Previous research in this project has shown that older rats frequently are unable to solve complex mazes which are easily mastered by younger animals. Analyses of the maze-running behavior of the older animals showed a striking tendency for these animals to make perseverative errors, i.e., a given animal tended to repeat its errors at the same point in the maze. However, the specific point in the maze where this occurred was idiosyncratic. Experiments completed this year have shown that it is possible to train all old rats in this task merely by preventing them from making errors on early trials. In fact, old rats so trained learn more proficiently (reach criterion in fewer trials) than do young rats.

It seems possible that the forcing of behavior in early trials in the animal study may be conceptually similar to breaking down the problem solving task into an analytical and a synthetic component. If so, the results from this study and the previously cited study in man are concordant. This interpretation is supported by the observation that both the rats and the men made perseverative or redundant errors. If these two findings are related, then it should be possible to investigate the mechanisms of perseverative errors in great detail using the rat model.

A study of learning and memory in man which was completed this

year also underscores the important, deleterious role played by perseverative errors. When subjects are required to recall information in a series of independent tests of recall, the older men show a tendency to make errors of intrusion, i.e., they are likely to recall information from a previous test and to intrude those data into the present test. This tendency for perseverative errors--which in exaggerated form occurs in severely brain-damaged patients--suggests that older subjects generally have a deficit in the ability to retrieve selectively newly acquired information. Nevertheless, as cited earlier, it is possible to devise learning procedures which effectively circumvent this problem.

The results of longitudinal (six year) analyses of performance on the Benton Revised Visual Retention Test, a measure of short-term memory for geometric figures, shows that longitudinal changes parallel cross-sectional ones. Subjects 40 years or less at the time of first testing show small increases in errors over a six-year interval; subjects in their 50s or 60s at time of first testing show a greater increase in errors, and subjects in their 70s at first testing show very large increases in errors of recall. These results are especially interesting because longitudinal age changes in performance on psychometric tests where time pressure is absent have rarely corresponded to results with cross-sectional tests.

A study of the effect of activity on longevity in mice was completed this year. Twenty-three month old male hybrid mice which were allowed ad lib access to an activity wheel for one month lived 10% (3 months) longer than did a matched control group. These differences were not due to weight loss among the active animals. Females did not show any gain in survival as a result of activity.

The clinical significance of visceral conditioning depends in part upon the ability of the subject to control his abnormal responses when he is away from the laboratory and in part upon his ability to maintain normal responses in the presence of external stresses. The evidence for control away from the laboratory comes from a number of studies done in this laboratory over the past several years in which it has been shown that patients with abnormal heart rhythms can reduce the prevalence of these rhythms as measured by continuous, 10-hour monitoring devices.

The evidence for the ability of subjects to maintain cardiac control in the face of external stressors was obtained from a study carried out with monkeys during this year. Each of four monkeys was trained to slow its heart rate, and each also was trained to appreciate a warning click which signaled an impending, inescapable electric shock and to discriminate this click from a neutral click not associated with shock. Training in these two procedures was given independently. The average increase in heart rate to the warning click was about 6 beats/minute and to the neutral click was 1 beat/minute. Blood pressure responses to the warning click were 9/7 mm Hg and to the neutral click were -2/-1 mm Hg. All of these responses were highly reliable. In the critical test of this study the clicks were sounded at a time when the monkey had to maintain slow heart rates. The question at issue was, "Would the animals slow their rates or would the click-stress prevail?" The results were clear. During the warning click period, heart rates fell about

19 beats/minute and during the neutral click period heart rates fell about 12 beats/minute. Thus the animals slowed their heart rates despite the inescapable stressor (shock). Interestingly, the monkeys continued to respond to the clicks with similar pressor responses (0/-1 mm Hg to the neutral click and 4/2 mm Hg to the warning click) even though they had reversed their cardiac responses.

In addition to the studies of the application of training techniques to the control of cardiovascular activity, a study was completed this year to evaluate the application of such training to the control of bowel function. A series of five adult and one child with histories of severe, intractable fecal incontinence (continuous daily soiling) were studied to determine if they could be taught to control anal sphincter activity. Four of the adult patients have achieved complete continence for periods ranging from one to three years, the 6-year-old child has remained continent for about two months, and the other patient has shown some improvement. Since the training was done on an outpatient basis and since patients could learn control quickly (two to three, one-hour sessions), it seems possible that the technique can be readily adapted as a simple means of treating some cases of fecal incontinence.

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Gerontology Research Center
Laboratory of Behavioral Sciences

There has been a significant reorganization within the Laboratory this year. The animal behavior program was transferred from the Psychophysiology Section to The Section on Human Learning and Problem Solving, and the name of the latter was changed to The Section on Learning and Problem Solving. The goal of this reorganization was to facilitate the scientific integration of these two programs, and this interaction has already begun to occur.

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Studies were initiated in the Psychophysiology Section this year to learn whether patients with essential hypertension could be taught to lower their blood pressures significantly. One patient has been studied this year. The patient was a 60 year old woman who had mild essential hypertention of at least 25 years duration. Her systolic blood pressure during the previous year was 147 mm Hg. (based on 15 clinic visits). After three weeks of training in the laboratory to control her blood pressure, the patient was instructed to take home blood pressures. Her average systolic blood pressure at home over the next three weeks (based on 25 determinations/day) was 127 mm Hg. Furthermore, this patient also was able to lower her systolic pressure voluntarily from this level to 110 mm Hg. (based on 25, self-administered trials/day over a three week period). These data are encouraging, however, with only one patient it is far too soon to draw any conclusions other than that the project is worth continuing.

The clinical significance of visceral conditioning depends in part upon the ability of the subject to control his abnormal responses away from the laboratory, and in part upon his ability to maintain normal responses in presence of external stresses. The evidence for control away from the laboratory comes from a number of studies done in this laboratory over the past several years in which it has been shown that patients with abnormal heart rhythms can reduce the prevelance of these rhythms as measured by

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Serial No. HD-AG7 (c)
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Verbal Learning and Age

Previous Serial Number: Same

Principal Investigators: David Arenberg, Ph.D. (50%)
Elizabeth A. Robertson, Ph.D. (68%)
(E.O.D. 9-1-72)

Other Investigators: Phillip Thorne
Richard Allen, Ph.D.

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	2.58
Professional:	1.18
Others:	1.40

Project Description:

Objectives: General objectives are to discover conditions which affect age differences in acquisition and retention of verbal material in man for two purposes: (1) an understanding of the factors which enable us to modify age differences experimentally should provide valuable information about aging processes and about psychological processes of learning; and (2) an understanding of the conditions which are particularly beneficial or deleterious to learning by the old may have implications for training old workers and education for the elderly.

Method Employed: Experiment XV was an age study of free recall in which lists of words were presented under three different conditions: (1) visual only, (2) visual plus passive auditory augmentation (the experimenter read each word aloud as it was presented), and (3) visual plus active auditory augmentation (the subject said each word aloud as it was presented). In free recall, the task is to recall as many words as one can after one presentation of the list. A similar study of free recall and age was conducted by Dr. Robertson in another laboratory (Experiment XVa). In that study, the auditory and visual presentations were independent; i.e., each list was presented in one modality.

Experiment XVII is a study of aging and encoding and decoding aspects of memory. Ten, three-letter items are presented under one of three conditions (decoding uncertainty) for each subject, and the task is to recall the three-letter items. The three letters in each item can be encoded into a word by three different rules. Each subject is instructed about one of the rules which determines a particular level of decoding uncertainty. For example, the three-letter item "UFN" can be encoded into the word "FUN" by the following three encoding rules: (1) reverse the first two letters; (2) place the vowel in the middle, or (3) rearrange the letters. These three encoding rules provide different amounts of information for decoding the word to recall the original three-letter item: (1) no decoding uncertainty--only one possible decoding according to the encoding rule; (2) low decoding uncertainty--two decodings possible; and (3) high decoding uncertainty--five decodings possible. After the ten items are presented, subjects are required to recall the letter groups and then the words; the number of words recalled and items recalled are the dependent measures. The experiment was designed to study the effects of decoding uncertainty on the recall of the letter-items and the words for five age groups of educated men.

Experiment XXI is another free recall study; it was designed to specify the source of interference discovered in Experiment XV. In that earlier study, it was found that recall of words presented at the beginning of a list is impaired by hearing or saying all the words in a list. Similar results were found in another study of free recall in which lists were presented by audio tape or by slides. Although auditory presentations resulted in more words recalled overall, fewer words were recalled from the beginning of the list than for visual presentations. In a pilot study using mixed lists, an attempt was made to determine for words near the beginning of a list whether saying interferes with recall of the spoken word or with recall of previous words in the list. It was found that when subjects were required to say only designated words (and look at the others), the "say" words were so compelling that few "look" words could be recalled. As a result, the interference effect the study was designed to investigate was entirely masked. Therefore, the procedure was modified. In the new study, the experimenter says specified words to provide the systematic variation of "look" and "listen" words. If this procedure does not permit the original effect to emerge, it may be necessary to use mixed lists of auditory and visual presentations rather than auditory augmentation.

Experiment XXIV is a new study of recognition memory and aging. In a recognition memory task, information is presented to an individual and he is subsequently required to distinguish items previously presented from a larger sample of items. Recent studies in the literature indicate that, under some conditions, age differences are not found for recognition memory. This study was designed to investigate two types of recognition procedures to determine whether age differences would be found. In addition, the results should provide information about whether there is an age deficit in temporal discrimination in a recognition memory task. Two recognitions tasks are used with auditory or visual presentation of the words at either a fast (1 sec. per word) or a slow (4 sec. per word) rate. In both tasks, three nine-word lists are presented, and then a print-out of these 27 words as well as nine other "distractor" words is immediately given to a

subject. In one task (recognition), the subject is asked to underline the words which were presented. In the other task (list discrimination), the subject is asked to designate to which list a word "belongs" by writing a "1" beside the word if it was in the first list, a "2" if it was in the second, a "3" in the third, and a "0" if the word had not been presented. The primary dependent measure is the number of correct responses adjusted for guessing. Each individual performs both of these recognition memory tasks under both input modality conditions at one level of the presentation rate variable.

Experiment XXV is a new study of short-term recognition memory using signal detection procedures. On the basis of recent empirical data using signal detection methods, a mathematical model has been developed which provides measures of acquisition and decay for individual subjects. One such study comparing young and old adults has been conducted in collaboration with Dr. Richard Allen of Baltimore City Hospitals; visual presentation of digits was used. In Experiment XXV, auditory as well as visual presentation will be used because under some conditions, the elderly benefit more than the young from auditory inputs in memory performance. Recent studies have also shown ear differences which have been attributed to hemispheric differences in the brain. An exploration of the acquisition and decay paremeters for the two ears for subjects of different ages may provide some information about relative age deficits for the two hemispheres. In addition, laterality differences in memory may be related to how subjects handle information dichotically, i.e., when different information is presented simultaneously to the two ears. An earlier study (Experiment XXII) in this project showed that some people handle such information ear by ear (sequential processing) whereas others handle it by pairs as presented (simultaneous processing). The approach (sequential or simultaneous) and the effectiveness (memory performance) may be related to the relative effectiveness of the two ears in short-term memory. In these recognition memory tasks, a series of items (digits, letters, words) are presented followed by a cue item. The task is to indicate whether the cue item had been presented in the series, and a certainty rating is made for each response. The acquisition and decay perimeters are obtained by plotting a signal detection measure of memory against time (presented position). The slope of the line provides a measure of decay, and the intercept estimates strength of acquisition at time zero (at input before decay begins).

Major Findings: Analyses of errors in Experiments XV and XVa, studies of free recall using auditory and visual presentations, showed a rather unexpected result. In this task, old subjects committed more errors than young adults. Just the opposite has been found in rote learning tasks (in which lists are presented many times); omission errors characterize the performance of the old learner in such tasks, and frequency of commission errors tends to be low. The difference between the rote and the free-recall procedures which probably accounts for the differences in frequency of commission errors is the feedback. In rote tasks, a subject's responses are immediately confirmed (or disconfirmed) and he knows immediately whether he is right or wrong. For the old learner, it is likely that an omission error is more acceptable than a commission error. In free recall, no feedback is provided, and the increase

in commission errors with age probably represents a retrieval deficit. In Experiment XVa, the commission errors were further analyzed, and it was found, in all four modality-rate conditions, that the old made more intrusion errors (words from previous lists) than the young (See Table 1). Of particular interest are the intrusion errors of words which were not recalled at the appropriate time (when the list which included that word had been presented). This means that the word was available (in store) but not accessible at the time it was supposed to be recalled. The fact that, in all four modality-rate conditions, old subjects commit such errors more frequently than young adults is further evidence of a retrieval deficit with age and also suggests that the temporal cues associated with those words have been lost (See Table 2).

Experiment XVII is the memory study in which letter groups are encoded into words and later decoded back to the original order of letters. Preliminary results reported last year indicated that the effect of the encoding rule (which produces variations in decoding uncertainty) affects performance in recall of the letter groups for all five age groups, as predicted; but the age effect was consistently high only for the seventy-year-old group. The sample is now sufficiently large to look at the performance measures for subjects who did not comply with the instructions. They were categorized into those who recalled the words first (letter groups are supposed to be recalled before the words) and those who did not form words to help them remember the letter groups (See Table 3). For those who recalled the words first, more words were recalled; but for the two conditions with some decoding uncertainty, fewer letter groups were recalled, as would be expected from the response interference of writing the words first. The data are sparse, but it looks as if the men over 60 are particularly susceptible fo the response interference when there is some decoding uncertainty. Age declines are emerging for all three conditions for those subjects who did not form words to help them remember the letter groups. It is also interesting to note that for those subjects, for zero decoding uncertainty, fewer letter groups were recalled than for those who used words as instructed; but the reverse was true for high decoding uncertainty. In other words, for the decoding condition which provides little help in remembering the letter-groups, fewer letter groups were recalled by those who used that approach than by those who used their own method; this was particularly evident for groups under 50.

Experiment XXI is the most recent study of free recall and age using the new procedure for mixed lists. In the new procedure, some words are read aloud by the experimenter as they are displayed, and other words are only displayed visually. Data collection was initiated using the new "listen" procedure, but the samples are too small to look at preliminary results.

Experiment XXIV is a new study of word recognition. Data collection has progressed sufficiently to show age differences emerging, but the effects of modality, rate, and type of recognition are not yet clear. (See Table 4)

Experiment XXV is a new study of recognition memory using signal detection procedures. Data collection is expected to begin by the end of the reporting year.

Significance to Bio-Medical Research and the Program of the Institute: Learning is more central to experimental psychology than any other behavior of higher organisms and some of the most striking and consistently reported behavioral age differences in the gerontological literature have been found in verbal learning performance. The experiments in this project are designed to identify basic mechanisms of learning and retention and to measure changes in these functions that occur with age.

Aging, viewed as a natural experiment in which many changes occur, permits one to identify a variety of processes which may be important underlying mechanisms of learning. In addition, knowledge about experimental variables which affect age differences will be valuable in developing techniques for optimizing learning of the older person.

Proposed Course of Project: Two manuscripts are being prepared for publication for Experiments XV and XVa. The sample for Experiment XVII will be completed during the next reporting year, data will be analyzed, and a manuscript will be written. Data collection will continue for Experiments XXI, XXIV, and XXV. Longitudinal results for serial and paired-associate learning (Experiments I and II) and visual retention of geometric figures will also be reported. The relations between the changes in these measures and blood pressure will be analyzed and reported. New studies of dichotic listening and tachistoscopic presentations (visual) will be initiated to study memory and modes of processing (sequential vs. simultaneous processing and effects of modality).

Honors and Awards: Drs. Arenberg and Robertson were invited to write the first chapter of a book entitled "Education for Aging" edited by Drs. Mason and Grabowski; the chapter is entitled "The Older Person as a Learner."

Publications: None

TABLE 1. MEAN NUMBER OF TOTAL INTER-LIST INTRUSIONS

(Experiment XVa)

		MODALITY	
	AGE	AUDITION	VISION
FAST RATE (1"/WORD)	20-39	4.10	6.40
	40-59	6.20	12.50
	60-79	10.80	16.60
SLOW RATE (4"/WORD)	20-39	2.30	3.50
	40-59	5.30	9.80
	60-79	10.50	12.00

TABLE 3. DECODING MEANS - APRIL, 1973 (EXPERIMENT XVII)

	NO UNCERTAINTY			LOW UNCERTAINTY			HIGH UNCERTAINTY		
	N	ITEMS	WORDS	N	ITEMS	WORDS	N	ITEMS	WORDS
UNDER 40	16	8.5	8.5	15	5.5	7.5	14	5.1	7.8
40's	24	7.5	8.1	18	5.1	6.8	19	4.3	6.9
50's	23	7.3	7.6	24	5.3	7.2	22	4.9	7.3
60's	19	7.3	7.5	21	5.1	6.9	16	3.7	6.3
70's	21	6.2	6.9	21	3.8	6.0	20	3.4	6.6
NOUNS FIRST									
UNDER 50	7	8.0	8.1	13	6.0	8.4	6	2.7	6.7
50's	12	7.6	8.8	8	3.9	7.6	4	5.5	8.0
OVER 60	14	7.5	8.2	6	2.7	8.0	6	3.0	8.3
ANOTHER SYSTEM									
UNDER 50	5	6.6	----	8	6.1	-----	24	6.1	-----
50's	7	5.3	----	6	5.0	-----	13	5.6	-----
OVER 60	8	4.7	----	11	4.5	-----	11	3.8	-----

TABLE 4. MEAN NUMBER OF CORRECT RESPONSES PER BLOCK

(EXPERIMENT XXIV)

AGE	RECOGNITION	LIST DISCRIMINATION
20-39	$\bar{X}$= 21.96	$\bar{X}$= 11.79
40-59	$\bar{X}$= 21.50	$\bar{X}$= 11.21
60+	$\bar{X}$= 19.10	$\bar{X}$= 8.85

TABLE 2. MEAN NUMBER OF TOTAL INTER-LIST INTRUSIONS NOT PREVIOUSLY RECALLED ("RETRIEVAL" ERRORS) (EXPERIMENT XVa)

		MODALITY	
	AGE	AUDITION	VISION
FAST RATE (1"/WORD)	20-39	0.80	1.10
	40-59	1.30	4.50
	60-79	3.60	5.00
SLOW RATE (4"/WORD)	20-39	0.40	0.40
	40-59	0.60	1.50
	60-79	2.40	3.80

Serial No. HD-AG8 (c)

1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland 21224

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Perceptual Retention and Age

Previous Serial Number: Same

Principal Investigators: David Arenberg, Ph.D.
Elizabeth A. Robertson, Ph.D. (15%)
(EOD 9-1-72)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	0.25
Professional:	0.15
Others:	0.10

Project Description:

Objectives: The general objectives are to investigate the effects of interference in perceptual retention and in perception: (1) to determine whether aging results in increased susceptibility to interference; (2) to explore conditions which affect age differences in interference; and (3) to hypothesize and develop procedures for testing mechanisms which may account for the empirical findings.

Methods Employed: In Experiment V, as in all previous experiments in this project, the basic retention paradigm consists of three sets of stimulus presentations: initial, interpolated, and final. A light or sound on for a specific period of time is a stimulus presentation and the subject is required to identify different durations by the labels that have been provided. The periods the light can be on in the initial and final sets are 0.20, 0.35, 0.62, and 1.10 sec. The four periods are verbally labeled "one," "two," "three," and "four" in order, and eight practice judgments are confirmed or corrected. This is followed by the primary task of the initial set which is to identify 32 periods during which no feedback about the correctness of judgment is provided. Performance is measured by the number of incorrectly identified stimuli. The periods for the interpolated set are 1.10, 1.6., 2.14, and 2.68 sec. These are labeled, practice with feedback

is provided, and then interpolated stimuli are presented without feedback. The final set consists of 32 presentations of the periods used in the initial set, but no practice is provided. The primary data consist of the change in performance (number of incorrectly identified stimuli) from the initial to the final sets. The conditions of the experiment are determined by the procedures during the interpolated set. Previous experiments (I, II, & III) have shown consistently that errors increase more for old than for young men when the interpolated set consists of visually presented stimuli labeled "one," "two," "three," and "four." In a more recent study (Experiment IV), it was shown that at least part of the age difference in interference was due to confusion introduced by the use of the same labels for the interpolated and the other sets of stimuli. When the labels for the interpolated set were "four," "five," "six," and "seven," the age difference in interference was substantially reduced. In Experiment V, the labels used for the interpolated set and the modality (visual or auditory) of those stimuli were included as independent variables.

Experiment VI is a new age study of speed of information processing. Stimulus pairs are presented for brief fractions of a second and two matching tasks are included: one is based upon physical identity, the other nominal identity. Under the physical identity conditions, the task is to affirm pairs such as AA and aa, but reject pairs such as Aa. Under nominal identity conditions, the task is to affirm pairs such as Aa, but reject pairs such as Ab. It is known that response times for physical identity conditions are shorter than for nominal identity, and the additional time required for the latter is attributed to the additional information processing required to affirm or reject a nominal match. In Experiment VI, it is hypothesized not only that an old group will be slower on both tasks, but that the difference between performance on physical and nominal matching will be greater for an old group than for a group of young adults. In addition, the pairs are displayed in different visual fields to explore differences in laterality (hemishperes of the brain).

Major Findings: In Experiment V, interference is measured under four conditions for the interpolated task (and each subject performs under one condition): (1) visual--same labels; (2) visual--different labels; (3) auditory--same labels; and (4) auditory--different labels (See Table 1). The two visual conditions have been included in previous studies in this project (Experiments I, II, III, and IV), and preliminary results are similar to the earlier findings; i.e., the old show greater susceptibility to the interfering effects of the interpolated task, but the age difference is reduced when confusability is minimized by using different labels for the interpolated stimuli. The auditory conditions are included for the first time in Experiment V, and the results emerging are rather unusual. As expected, the young groups are slightly less affected by the auditory interference. It was predicted that auditory stimuli would interfere less with visual judgments than would visual stimuli. For the old, however, the opposite result is emerging. Under both auditory interpolated conditions, the old are showing more interference than under the visual interpolated conditions; and this modality difference is particularly striking for the high-confusability condition in which the set of interpolated stimuli have

the same labels as the set of primary stimuli.

Significance to Bio-Medical Research and the Program of the Institute: The general idea that, with age, a person becomes more susceptible to interference is well entrenched in gerontological thinking and is often used to "explain" age differences in performance. The evidence for this idea, however, is sparse. It is the purpose of this project to explore the generality of the age-interference hypothesis for both non-verbal memory and perception. It is important to know, for both theoretical and applied reasons, under which conditions the older individual is especially interference prone.

Proposed Course of Project: Data collection will continue for Experiment V and will be initiated for Experiment VI. Experiments planned in this project will explore age effects of more basic perceptual interference phenomena such as masking and disinhibition.

Honors and Awards: None

Publications: None

TABLE 1. MEAN ERRORS (EXPERIMENT V)

	VISUAL				AUDITORY			
	SAME		DIFFERENT		SAME		DIFFERENT	
	Young	Old	Young	Old	Young	Old	Young	Old
Initial Errors	6.32	10.13	6.48	7.25	8.22	7.00	8.22	8.38
Final Errors	10.89	16.50	10.33	12.44	12.28	17.88	11.39	14.50
Difference	4.57	6.37	3.85	5.19	4.06	10.88	3.17	6.12
N	19	8	21	16	18	8	18	8

Serial No. HD-AG6 (c)
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Problem Solving and Age

Previous Serial Number: Same

Principal Investigator: David Arenberg, Ph.D. (50%)
Leonard M. Giambra, Ph.D. (66% - E.O.D. 8-14-73)

Other Investigators: Phillip Thorne

Cooperating Units: Baltimore City Hospitals
Miami University of Ohio
College of Notre Dame of Maryland

Man Years:

Total:	2.46
Professional:	1.16
Others:	1.30

Project Description:

Objectives: The general goals are to explore and identify reasoning processes in man, to determine in what ways these processes change with age, and to develop techniques for reducing age deficits in reasoning performance. In this project, reasoning is studied by using problem-solving procedures in which on-going solution behavior can be observed and quantified. Experiments are designed to answer such questions as: (1) Is effectiveness in acquiring relevant information affected by aging? (2) Is effectiveness in synthesizing available information affected by aging? (3) What kinds of solution strategies are used and in what ways are they related to age? (4) How does imposing a memory load affect solution strategies for young and old adults?

Methods Employed: Experiments I and IV use the same equipment and the same problems, but the procedures are different. The display board for all problems includes nine lights on the periphery of a circle and a goal light in the center. The peripheral lights can be turned on by switches. In Experiment I, a subject's task is to light the goal light in a prescribed way. For each problem, a disk is superimposed on the light display panel. On the disk are arrows connecting various lights. An arrow indicates that one of three logical relations (previously explained to the subject) exists

between the lights that arrow connects, but the specific relation is not identified. The solution of each problem requires lighting the goal light by a sequence of inputs[a] in which only the switches for a specified set of three (of the nine) peripheral lights can be used. Time between inputs is controlled by the subject. In addition to correctness of the solution, performance is evaluated by determining whether an input (at the time it is made: (1) provides new information, (2) provides inferable information, or (3) is overtly redundant. In Experiment I, subjects are not required explicitly to identify the relation (meaning) of each arrow on the problem disk. This is the procedure typically used in these problems. A subject may reach a solution with great difficulty when one (or more) of his implicit identifications of the relations is incorrect. This will sometimes appear to be a decrement in synthesis performance, when in fact the difficulty should be attributed to the mis-identification which is an analysis error. Experiment IV was designed to avoid the confounding of analysis and synthesis performance. What is a one-task problem in Experiment I was transformed into a two-task problem. The first task is to identify the relations (the meanings of the arrows between lights). After this is completed, the subject is provided with all the correct identifications, and these are reviewed before he is permitted to work on the solution to the problem. As a result, synthesis performance, i.e., putting the information together to reach a solution, is always based upon accurate and complete information, and is uncontaminated by poor performance during analysis (identification of arrow meanings). In addition to correctness in each task, effectiveness of performance can also be measured. For the analysis task, one index of effectiveness is the number of correct identifications. For the synthesis task, all inputs other than the minimum required to attain the solution are superfluous. The number of superfluous inputs is an index of ineffectiveness in synthesis performance.

a. The term "input" refers to the situation in which one or more lights are on and a subject indicates he wants the results of those lights. Those lights are then extinguished, and according to the logical relations of the arrows pointing from those lights, the resultant lights are turned on.

Experiment VII is the most recent in a series of experiments designed to explore an unusual (but highly replicable) finding from cross-sectional analyses of the longitudinal study of concept identification (Experiment V). Under conditions of high initial information it was found in Experiment V that effectiveness in identifying disjunctive concepts was superior to effectiveness in identifying logically equivalent conjunctive concepts. Superior performance for disjunctive over conjunctive concepts is virtually unknown in the literature, but it was found for four independent age groups under the conditions of the longitudinal study. Attempts have since been made to identify variables and procedures which contributed to this unusual finding. In Experiment VII, the primary variable is what we have termed "linguistic congruence." In Experiment V, the positive designation is always "Died" which has negative linguistic properties. In Experiment VII, designations for half the sample are also linguistically incongruent, whereas for the other half the designations are congruent with their linguistic properties; e.g., "Well" is the positive designation and "Sick" is the

negative. Logically equivalent conjunctive and disjunctive concepts with high and low initial information gain are included.

Experiment VIII is a new concept-identification study which departs substantially from all the others in this project. The intent is to explore subjects' preferences between information expressed conjunctively and information expressed disjunctively when the choices are equivalent in information gain. It is possible to provide choices with equivalent (or non-equivalent) information gain by modifying the equivalence-of-information method developed in this Section. Two experiments have been designed in which each preference in a sequence is represented on two variables--conjunctive or disjunctive, and positive or negative; and the resultant vector of an entire sequence of preferences provides a preference summary. Each choice in the sequence to establish an overall preference is between two alternatives with equivalent information. In the first preference study, in addition to the preference problems, two types of performance problems were included. In one, four examples were presented simultaneously for each problem, with all designations consistent in one dimension, i.e., all four designations were conjunctive (two positive and two negative), all were disjunctive (two positive and two negative), all were positive (two conjunctive and two disjunctive), or all were negative (two conjunctive and two disjunctive). All four such problems are informationally equivalent, but shorter times to reach a correct solution were predicted when the consistent element was prevalent in the choices during the preference problems. In other words, it was predicted that a preference for a particular type of designation would be reflected in faster performance on problems with designations like those preferred than on problems with non-preferred designations. In the second type of performance problem, only one example with its designation was given, and the task was to assign all 24 pairs (solution possibilities) into those that were viable (consistent with the example) and those that were not viable (inconsistent with the example). Again it was predicted that performance would be related to preference. In addition to the preference and performance tasks, subjects were asked to rate the designations on informativeness, ease in handling the information (difficulty), and ease in keeping track of the information (memory).

Experiment IX consists of several new studies of concept identification in which the interval between examples is a variable. In all previous studies of concept identification in the project, the memory load was minimized by providing the subject with a written record of the examples and their designations throughout each problem (designations are feedback messages indicating whether an example is positive, i.e., exemplifies the concept, or negative, i.e., does not exemplify the concept). In Experiment IX, memory load is manipulated by including as variables time between examples, and the number of examples on display at any one time. For all of the studies, examples consist of displays with three "dimensions" and each dimension can assume one of three "values." The dimensions are number, color, and shape. The number can be one, two, or three; the color can be red, green, or black; the shape can be a circle, square, or plus sign. An example consists of a combination of a number, a color, and a shape. In the first study, only conjunctive concepts are used. Subjects are instructed that two dimensions

are relevant, but are not told that the concept rule is conjunction, i.e., two attributes must both be present for an example to be positive. The concept could be three and square, two and red, green and circle, or any of the 27 possible pairs of attributes. After each example, the subject responds with one of the two designations (positive or negative), and then the correct designation is presented. All 27 possible examples are presented sequentially, and the number of correct designations is the primary dependent measure. In the first study, the interval between examples is 1, 9, 17, or 25 seconds, and three problems are presented to investigate the learning factor. The other independent variables are age and sex.

Experiment X is another group of new concept studies, similar to Experiment IX, but instead of varying the interval between examples, the primary variable is the designation label. Instead of "positive" and "negative," or "yes" and "no," the feedback message can be "A" or "B," or "A" or "not A," for example. Other independent variables are age, sex, concept rule, and the effect of experience (comparing first, second, and third problems).

Major Findings: Preliminary results of Experiment IV (N=173), which is a modification of Experiment I (a study of logical problem solving), were quite similar to the cross-sectional results of the earlier study (See Table 1). The proportion of successful solutions declined with age; and for those who were able to solve a problem, the number of noninformative inputs increased with age. In addition, due to the procedural changes, it was possible to look at the analysis and synthesis parts of the problems separately in Experiment IV. In the analysis parts, very few errors were made in identifying arrow meanings by any age group. Although the number of noninformative inputs increased with age, the differences were small in analysis. Noninformative inputs can be either inferrable or overtly redundant. In Experiment I, a substantial portion of the age differences in noninformative inputs were attributable to the increase in overtly redundant inputs with age. The results of Experiment IV indicate that those overtly redundant inputs in Experiment I were synthesis difficulties and most likely represented incorrect solution attempts which were repeated in whole or in part.

Experiment VII is the concept-identification study in which linguistic congruence of the feedback messages were varied; i.e., the positive designation could have positive or negative linguistic properties. It was found that students from nearby high schools were inappropriate for this study, and data collection was initiated for a sample of college students.

Experiment VIII, the first study of concept preference, found no strong preferences among highly intelligent college students. It is likely that a less select sample of procedural changes requiring an additional memory load would yield preferences, especially if the performance was timed. Ratings on all three scales were found to divide examples quite consistently into high information and low information. Conjunctive positive examples and disjunctive negative examples are highly informative (permit the elimination of a large number of possible solutions), and they were rated not only more informative than conjunctive negative and disjunctive positive

examples, but easier to handle and easier to keep track of. No differences in ratings were found between the two highly informative examples or between the two low-information examples.

Data collection is about to begin for Experiment IX, a concept study in which a memory load is required and the interval between examples is varied.

In the first study in Experiment X (X_a), five pairs of designations were "A" and "B," "A" and "not A," "+" and "-," "*" and "⊕," or "Died" and "Lived." The concept rule was conjunction, disjunction, conditional, or biconditional. Data have been collected for all the young subjects (N=160), but analyses have not been completed.

Significance to Bio-Medical Research and the Program of the Institute: Reasoning is among the most prized behaviors of man and among the most elusive for experimental study. In this project, methods have been and will be developed for obtaining quantifiable measures of step-by-step performance on reasoning problems. Some of these methods also provide patterns of responses which represent strategies in solving such problems.

Measures are obtained in current experiments to study changes in reasoning processes with age. These studies, in addition to identifying basic reasoning processes, should indicate how pervasive reasoning deficits are with age, whether education and cognitive activity mitigate such deficits, and what techniques could be used to minimize decline in reasoning.

Proposed Course of Project: When the ten or twelve subjects who are due for a repeat session in Experiment I are seen this year, that longitudinal sample will be completed; and a manuscript describing that study will be prepared for publication. It will be the first such longitudinal study in the gerontological literature. Data collection will continue for Experiment IV, and that sample will be closed, at which time a manuscript will be prepared for publication. Data collection will continue for Experiments VII, IX, and X, and another concept-preference study will be designed to follow up Experiment VIII.

Honors and Awards: Dr. Arenberg was appointed chairman of the Program Committee for the Division on Adult Development and Aging of the American Psychological Association for its meetings in Montreal in August, 1973.

Publications: None

TABLE 1. MEANS OF PROBLEM SOLVING MEASURES (EXPERIMENT IV)

		ANALYSIS			SYNTHESIS		
	N	NO. CORRECT	NONIN-FORMATIVE	REDUN-DANT	PROPORTION SOLVED	SUPER-FLUOUS	REDUNDANT
PROBLEM I							
Under 40	21	10.3	1.95	.05	.95	2.30	1.00
40's	46	9.6	3.31	.24	.85	4.41	2.15
50's	44	10.2	3.30	.34	.91	5.48	2.47
60's	38	9.7	3.41	.35	.76	4.17	1.76
70's	38	9.5	4.36	.81	.68	4.46	1.88
80's	6	7.7	4.67	1.33	.00	----	----
PROBLEM II							
Under 40	20	9.8	1.72	.00	.90	1.72	0.44
40's	42	9.7	2.40	.03	.88	3.95	1.14
50's	40	9.5	2.60	.03	.88	4.17	1.51
60's	25	9.5	2.78	.04	.76	10.24	5.76
70's	26	9.2	3.95	.45	.54	9.43	3.71
80's	0	---	----	---	---	----	----

Serial No. HD-LBS-2

1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 - June 30, 1973

Project Title: Daydreaming and Aging

Previous Serial Number: None

Principal Investigator: Leonard M. Giambra, Ph.D. (22%; E.O.D. 8-14-72)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals
Community College of Baltimore
College of Notre Dame of Maryland
Miami University of Ohio

Man Years:

Total:	.45
Professional:	.22
Others:	.23

Project Description:

Objectives: To daydream is to have spontaneous thoughts that are relatively unrelated to the task at hand. Such thought processes are usually considered the preserve of the adolescent and young adult. The purpose of this study is to relate frequency and content of daydreams to age across the entire adult range. With changes of age come changes in attitudes, responsibilities, life style, experiences, potentialities, leisure time, demands on the individual's attention, and physiology. It is hypothesized that the examination of daydreaming tendencies of various ages will reveal the effect of these age related variables upon the thinking processes of individuals. Thus the primary objective of this project is to examine daydreaming tendencies to determine age differences and changes in this type of thinking process.

Methods Employed: The daydreaming tendencies of every participant in this project is obtained through self report via the Imaginal Processes Inventory developed and copyrighted by Jerome L. Singer and John S. Antrobus. The Imaginal Processes Inventory (IPI) is a 344 item questionnaire which has both specific and general items concerning daydreams, night dreams, fantasy, etc. There are two groups that are contributing to the data pool.

The first group consists of volunteers recruited in the community and at colleges and universities. The second group consists of the participants in the Baltimore Longitudinal Study of the GRC. The first group consists of individuals from a wide variety of ages, sex, income, race, religion, ethnic background, and socioeconomic class. The longitudinal group is all male and relatively homogeneous with regard to income, race, religion, ethnic background and socioeconomic class. Both groups allow for a cross-sectional comparison of age differences in daydreaming while only the longitudinal group allows for a truly longitudinal determination of age related change (if any). Since this project began in December of 1972, no longitudinal information is available.

A total of 135 males were divided into five age groups: 17-22 (n=88), 27-35 (n=8), 38-49 (n=11), 51-62 (n=18), and 66-77 (n=9). The youngest age group was made up of students of Miami University. The remaining groups were composed of participants in the Longitudinal Study of the Gerontology Research Center of the National Institute of Child Health and Development and represent the first portion of a continuing effort.

Major Findings: Since this must be a brief report and since the number of Ss was relatively small, the results of the questionnaire will be portrayed by looking at only some selected items. Some of these items along with the proportion of each age group selecting each items' five alternatives are given in Table 1.

Table 1 goes about here

Attitudes toward daydreaming - Item 177 indicates that at least 72% of every age group considers daydreaming in a positive light; furthermore that the youngest group has the most negative feelings toward daydreaming. It would be reasonable to conclude that the Ss would be honestly answering the questionnaire since daydreaming appears to be a socially desirable behavior which one would not conceal.

Daydreaming frequency/duration - Items 1, 2, and 3 are pertinent. Item 1 indicates that daydream frequency is a maximum at ages 17-22 and slowly decreases to the 38-49 age where it levels off into the 51-62 age then slightly decreases for the 66-77 age. Note that even at age 66-77 daydreaming occurs a few times or more a day for 66% of the group. Item 2 indicates that the percent of waking thoughts in daydreams or fantasies parallels frequency for the five age groups except that the 66-77 age group tends to devote a greater percentage of time than the 51-62 age group. Item 3 parallels item 2. One might conclude that daydreaming does steadily decline with age but that with retirement age there is a slight increase of daydreaming. Most importantly, however, one must conclude that for the majority daydreaming continues throughout life.

Time orientation of daydreams - Items 28, 68, 69, 99, 146, 239, and 295 are pertinent here. It is commonly believed that most daydreams are future oriented and with increasing age (and a decreasing future) that future oriented daydreams would diminish and past-oriented daydreams would increase. Daydreams about "different ways of finishing things I still have to do in my life" were highest for the two youngest groups and lowest for the 38-49 group with a resurgence toward the higher levels for the two oldest age groups. Reliving "happy or exciting experiences in my daydreams" was highest for the youngest group and sharply declined for the 27-35 group followed by a monotonic decline for the remaining age groups. Daydreams "closely related to problems that come up in daily life" occurred most frequently with the youngest group and least often with the eldest group; the ages between showed a decline except for a resurgence at 38-49. Daydreams which are "so much like the things I do from day-to-day" occurred most often in the 38-49 age span and declined with decreases or increases of age except that the eldest group had a greater tendency than the second eldest. Events which happened over a year ago were most often in the daydreams of the eldest closely followed by the youngest group; this type of daydream occurred least often in the 27-35 and 51-62 age spans with the 38-49 age span near minimum. The greatest concern with the present occurred in the daydreams of the youngest and eldest age groups with the 51-62 group showing the least concern. These results were not completely consistent but it appears that a past orientation in daydreams predominates in both the youngest and oldest age groups with the middle age groups (especially the 38-49) being present oriented in their daydreams. It is true however that the tendency to have daydreams about the less recent past was weakest in the youngest group with a gain in strength with increasing age until maximum with the eldest group. Finally one must conclude that looking toward the future in daydreams is an important and pervasive orientation of daydreaming in the elderly.

Daydreams regarding personal expectations - Items 57, 115, and 199 are pertinent. Daydreams about exceeding parents' expectations were at a high level for the youngest group; for the other age groups such daydreams were at a relatively low level finally dropping to near zero for the 66-77 age group. Daydreaming about being promoted to a better position was highest for the 17-22 age span and lowest for the 66-77 age span with a steady decline between. The tendency to imagine oneself as attractive to the opposite sex was about equally strongest at 17-22 and 27-35 followed by a diminuation with succeeding ages to a minimum for the eldest. Essentially, then increasing age brings with it a decreasing frequency of daydreams which are achievement specific, perhaps brought about by the esteem deflating experiences which are likely to pile up with age.

Daydreams about impossible and heroic things - Daydreaming about "utterly impossible situations" were uncommon at all ages with the 17-22 age span showing the greatest tendency and the 27-35 age span the least. Items 270 and 317 are about heroic daydreams and parallel one another. The tendency was strongest in the youngest group with a sharp decline for the 27-35 age group followed by a further decline to a minimum in the 38-49 and 51-62 age spans followed by a small increase with the 66-77 age span. It would seem that the post-school jump in responsibilities, a job, and family would account for this.

TABLE I

The Proportion of Each Age Group Selecting Each Alternative of Selected Items

Item Number and Item	Age (in years)				
	17-22	27-35	38-49	51-62	66-77
2. Daydreams or fantasies make up:					
(a) No part of my waking thoughts	0	0	0	11	11
(b) Less than 10% of my waking thoughts	27	37	45	61	44
(c) At least 10% of my waking thoughts	39	37	45	22	22
(d) At least 25% of my waking thoughts	26	25	9	5	22
(e) At lest 50% of my waking thoughts	6	0	0	0	0
3. I would describe myself as:					
(a) Someone who never daydreams	0	0	0	5	0
(b) Someone who very rar ly daydreams	4	12	18	11	22
(c) Someone who tends toward occasional daydreaming	31	50	63	50	33
(d) Someone who tends toward moderate daydreaming	48	25	18	27	33
(e) A habitual daydreamer	14	12	0	5	11
57. In my daydreams, I exceed my parents' expectation:					
(a) Definitely not true	2	0	36	47	50
(b) Usually not true	42	62	54	23	50
(c) Usually true	29	37	9	17	0
(d) True	18	0	0	11	0
(e) Very true	7	0	0	0	0
68. I often relive happy or exciting experiences in my daydreams:					
(a) Definitely not true	3	12	0	17	12
(b) Usually not true	9	25	36	35	50
(c) Usually true	23	50	54	35	25
(d) True	35	12	9	11	12
(e) Very true	28	0	0	0	0
115. I daydream about being promoted to a better position:					
(a) Definitely not true	12	12	27	41	25
(b) Usually not true	44	50	54	29	62
(c) Usually true	19	25	18	23	12
(d) True	20	12	0	5	0
(e) Very true	3	0	0	0	0

Item Number and Item	Age (in years)				
	17-22	27-35	38-49	51-62	66-77
146. I often daydream about events that happened over a year ago:					
(a) Definitely not true	3	0	9	17	0
(b) Usually not true	32	87	45	58	25
(c) Usually true	31	0	45	11	50
(d) True	23	12	0	11	25
(e) Very true	7	0	0	0	0
239. I daydream more about events that have already happened than about things in the future:					
(a) Definitely not true	5	12	9	5	0
(b) Usually not true	47	50	54	52	62
(c) Usually true	29	25	27	35	0
(d) True	12	12	9	5	37
(e) Very true	4	0	0	0	0
270. I picture myself risking my life to save someone I love:					
(a) Definitely not true	2	12	36	33	37
(b) Usually not true	22	62	45	44	25
(c) Usually true	32	25	18	16	37
(d) True	28	0	0	5	0
(e) Very true	13	0	0	0	0

Significance to Bio-Medical Research and Program of the Institute: The study of daydreaming is fundamentally a study of thought processes. Furthermore there has been little research in this area of thought processes especially across the entire adult age range. In order to understand fully the thought processes of man, the total spectrum of those processes need to be examined. In addition, one must know how this wide spectrum is affected by aging. Thus the study of daydreaming in adults, along with other variables such as differences in age, socioeconomic status, attitudes, etc. may help us understand the fundamental processes which underly them all.

Proposed Course of Project: Data collection will be continued from the community to broaden the population base and from the longitudinal participants on their next and subsequent visits to obtain measures of changes with age.

Honors and Awards: Dr. Giambra was invited to lecture to the members of the Psychology Club of Loyola College (December, 1972), to the members of the Senior Citizens Group (Bykota House) in Towson, Maryland (February, 1972), and to the Students of Community College of Baltimore (April, 1973).

Publications: Giambra, L.M. Daydreaming in males from seventeen to seventy-seven: A preliminary report. Proceedings of the 1973 Convention of the American Psychological Association (in press).

Serial No. HD-LBS-1

1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report

July 1, 1972 through June 30, 1973

Project Title: Behavioral Genetics and Aging.

Previous Serial Number: None

Principal Investigator: Charles L. Goodrick, Ph.D. (40%)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.80
Professional:	.80
Others	.00

Project Description:

Objectives: The principal objectives of this project are (1) to determine group differences for behavioral traits and longevity among inbred strains of mice; (2) to determine: (a) heritability (degree of genetic determination vs. degree of environmental determination); (b) mode of inheritance (e.g. over-dominant, dominant, intermediate, or recessive); and (c) number of segregating units (gene blocks) controlling a particular trait (e.g. longevity); and (3) to examine relative behavioral differences among mouse strains as aging progresses.

Other objectives include analyses of the influence of diet (e.g. protein available) on behavioral traits, growth, and longevity; and determination of single-gene influences upon behavioral traits, growth, and longevity.

Methods Employed: Inbred mice(C57BL/6J and A/J)of a high degree of homozygosity are maintained under uniform environmental conditions. The animals are tested behaviorally during one period of their life span, e.g., when mature, mature-old, or aged. Old age is determined as the 50% mortality point for groups maintained throughout their life span. Statistically reliable techniques have been developed to determine behaviors relevant to natural selection such as exploration, general activity level, emotionality, simple or complex problem solving ability and taste preference. The use of

segregating groups allows an estimate of the mode of inheritance, e.g., dominant or intermediate, and the number of gene blocks or segregating units controlling behavioral traits or life span. The diets used for studies in which protein intake is varied among groups of inbred and hybrid mice are isocaloric synthetic diets obtained from General Biochemicals, Chagrin Falls, Ohio. Deprived animals receive 4% casein in their diets whereas the control group receives a 26% casein diet. Numerous kinds of mutant mice are maintained on the C57BL/6J background at the Jackson Memorial Laboratories; Bar Harbor, Maine. Our work has concentrated on the albino, beige, yellow and obese mutations.

Major Findings:

A. An experiment has been completed to determine the effects of aging and genetic constitution upon behavior and longevity for A/J and C57BL/6J inbred mice. A/J mice are albino (white coat), short lived behaviorally inactive mice, while C57BL/6J mice are melanic (black coat), long lived, behaviorally active mice. Both groups die of nonspecific causes. Groups of A/J, C57BL/6J, F_1 and F_2 mice have been tested on a number of behavioral measures with an N at least 16 per group at 5, 14, and 23 months of age, and at the time of advanced old age for the four groups: A/J (23 mo.) C57BL/6J (26 mo.), F_1 and F_2 (29 mo.) at the approximate time of senility or 50% mortality. Behavioral data were collected using standard tests developed in our laboratory to determine: (1) sensory discrimination, (2) preference for ethyl alcohol, (3) performance and retention of a learned operant response, (4) exploration behavior, and (5) emotionality. These data are in the process of analysis.

B. An experiment was initiated to examine the generality of mode of inheritance for the behavioral traits of exploration behavior, emotionality, and alcohol preference using mature-young mice of four inbred strains. The inbred strains were A/J, BALB/cJ, C57BL/6J, and DBA/2J, while the hybrid groups were AxB, AxC, AxD, BxC, BxD, and CxD, and both males and females were tested for each of the ten groups (total N=360). This experiment was necessary because one of the major drawbacks to our studies of aging and genetics using A/J and C57BL/6J mice was the possible lack of generality for any results obtained. For exploration behavior, it was hypothesized that exploration activity is inherited in the same manner for all six F_1 hybrid groups, and the specific mode of inheritance is intermediate. A diallel genetic analysis of combining abilities has been completed for this measure. This analysis provides strong evidence that the general mode of inheritance is similar for all F_1 groups because GCA (general combining ability) was statistically significant for both male and female groups, while SCA (specific combining ability, indicative of differing modes of inheritance), was not statistically significant for male or female groups. The mode of inheritance was intermediate as hypothesized.

C. A number of groups of mice are being currently maintained to obtain mortality data. Our early studies used A/J, C57BL/6J, F_1, and F_2 groups for studies of behavioral differences for populations which differ with respect

to genetic constitution and age. In addition to behavioral measures, 100 animals for each group were used to determine average longevity. In order to obtain additional information and determine the possible generality of our earlier findings, we are maintaining populations of A/J, BALB/cJ, C57BL/6J, and DBA/2J inbred mice, and the six possible F_1 hybrid groups. This study will yield information with respect to longevity of these inbred strains, and additional information with respect to the mode of inheritance of longevity. Groups of four mouse mutations with littermate controls are also being maintained to determine longevity, all on the C57BL/6J background. These four mutations (Bg, A, Y, and ob) all differ from controls on the major dimension of body weight. Further data are also being obtained with regard to the body weight and longevity of A/J, C57BL/6J, and F_1 hybrid groups fed isocaloric synthetic diets which differ in protein content. These longevity projects will continue for an additional 24 months.

D. A series of experiments is in progress concerned with growth, longevity, and behavior as a function of level of dietary protein. An experiment has been completed that was designed to determine the effect of environment and dietary protein upon growth and behavior of inbred and hybrid mice. The subjects were A/J, C57, and F_1 hybrid mice (N=300, 100/group). At 5-weeks of age the mice were weighed and placed on either a low or normal protein diet (N=50 low protein and N=50 normal protein for each group). The average weight for each group at this time did not deviate significantly from 15 grams. The test diets were synthetic, isocaloric diets which contained either 26% casein or 4% casein as the source of protein. Each subgroup (N=50) was housed in small suspended cages (1 per unit, N=14; 2 per unit, N=16) or large clear plastic cages with 5 mice per cage. In addition to measures of body weight, after the age of 15 weeks each mouse was given 2 tests, one of forced exploration and emotionality, the other of free exploration and emotionality. The major results of this experiment were the following. Group differences in weight were highly statistically significant, F (2,234)= 41.95, p <.01. A/J mice were lowest in body weight, C57BL/6J mice were intermediate in body weight, and F_1 mice had the highest body weight. Mice maintained on low protein diets had lower mean body weight than mice maintained on normal protein diets, F (1,234)= 840.18, p <.01. The Group x Diet interaction was statistically significant, F (2,234)= 5.63, p <.01, because differences between low and normal protein groups were less for A/J mice than for C57BL/6J or F_1 mice. The interaction of Diet x Caging was also statistically significant, F (2,234)= 24.43, p <.01. This interaction was due to an effect which occurred for all three mouse groups. For normal protein groups, mean body weight was greater for mice which were group caged than for isolated mice, while for low protein groups, mean body weight was lower for mice which were group caged than for isolated mice.

For forced exploration scores, group differences were highly statistically significant, F (2,200)= 637.54, p <.01, with A/J scores low, C57BL/6J scores high, and F_1 scores intermediate. Groups given low protein diets had higher scores than groups given normal protein diets, F (1,200)=4.75, p <.05, and mice which were group caged engaged in less exploration than singly caged mice, F (2,200) = 17.97, p <.01.

The Group x Diet interaction was statistically significant, $F(2,200)= 23.08$, $p < .01$, due to larger differences for A/J than for F_1 mice and a reversal for C57BL/6J groups as a function of diet. All low protein groups of A/J and F_1 mice had higher activity scores than normal protein groups, while C57BL/6J normal protein groups all had higher scores than low protein groups. An interaction was obtained between Group and Caging F $(4,200)= 4.56$, $p < .01$. The decrease in exploration as a function of increasing number of mice per cage was greatest for the C57BL/6J group, smaller for the F_1 group, and not obtained for the A/J group. Scores for all F_1 groups were not significantly different from the midparental values $\left(\frac{P1 + P2}{2}\right)$.

For the analysis of scores of free exploration (C57 and F_1), both main effects of Group and Diet and their interaction were statistically significant, Group x Diet F $(1,132)= 28.33$, $p < .01$, Table 1. Hybrid groups fed low protein diets had significantly higher free exploration scores than hybrid groups fed normal protein diets, but C57 dietary groups were not significantly different. Caging was not a significant variable for this test, and the other interactions were not statistically significant. Although the F_1 normal protein groups all obtained mean scores intermediate in MI, the MI for all low protein groups was partial dominance, with means for groups caged 1 or 5 per cage significantly greater ($p < .05$) than midparental values.

Analyses of emotionality scores obtained during both exploration tests (free and forced, A/J and F_1 only) indicated that open field emotionality was greater with increasing numbers in the cage; for forced exploration, caging, F $(2,132)= 13.78$, $p < .01$; for free exploration, caging, F $(2,132)= 11.61$, $p < .01$. The same effect of caging was obtained for both C 57 groups fed normal protein. Diet was also a significant main effect for both tests. Mice maintained on low protein diets had lower scores of emotionality than mice maintained on normal protein diets, Diet (forced exploration), F $(1,132)= 13.77$, $p < .01$, (free exploration) F $(1,132) = 26.53$, $p < .01$.

During tests of C57 mice fed low protein diets, 65 of 72 scores were zero, while 31 of 72 scores were zero for C57 mice fed normal protein diets. For emotionality scores during the free exploration test, the second order interaction attained statistical significance because F_1 scores for low protein groups housed 1 or 2 per cage were significantly lower than scores of A/J groups, while all other comparisons did not differ significantly. All F_1 groups, with the exception of the two above, scored significantly higher than the midparental values, but were not significantly different from the higher scoring parental value. This experiment supplements previous research which obtained the same basic behavioral differences between mice fed low or normal protein diets, and in addition examined the effects of environmental complexity or group caging.

E. Water intake, alcohol intake, and alcohol preference also were determined for mice fed low (4%) or normal (26%) percentages of protein in isocaloric synthetic diets. A/J, C57, and hybrid male mice 6-months of age were subjects (N=240). Mice which are fed Purina rodent chow were also tested. The protein content of Purina chow is about 25%, but the concentration of carbohydrate (in this case, sugar) is much less than that of the normal

protein synthetic diet. When the mice given Purina chow were tested for alcohol preference, the A/J strain avoided the alcohol, the C57 strain preferred alcohol, and the hybrid groups were intermediate in preference for alcohol. Fluid intake was similar for all groups, from 4 to 5 ml. per day, and group differences were consistent for all alcohol concentrations. Mice given synthetic diets which differed in protein content (Normal protein = 26%, low protein = 4%) differed with respect to the above control data in both alcohol preference and total fluid intake. At a moderate alcohol concentration of 5%, normal protein groups obtained scores similar to those of groups fed Purina chow, while low protein groups all obtained similar preference mean scores of over 50%. The total alcohol intake was higher for all low protein groups than for normal protein groups because total fluid intake was greater for low protein than for normal protein groups. At the higher alcohol concentration of 10%, there was a reduced alcohol preference for both protein levels. This effect of an aversion to the high alcohol concentration was greater for low than for normal protein groups. The C57 group fed Purina chow obtained very high alcohol preference values at the 10% concentrations, compared with groups fed low or normal protein synthetic diets. There are two findings of major importance with respect to this research: (a) the reactivity to moderate levels of this drug was greater for mice fed low protein diets than for mice fed normal protein diets, (b) for C57 mice alcohol preference for the high concentration was greatly reduced for animals fed synthetic diets compared with the preference of controls fed Purina chow. Some feature of the synthetic diet resulted in a marked reduction in alcohol preference for animals which normally highly prefer alcohol solutions.

Significance to Bio-Medical Research and Program for the Institute: The study of the genetics of behavior and longevity allows an assessment of: (1) the mode of inheritance for the factor studied (i.e., dominant, intermediate, etc.); (2) the relative importance of hereditary and environmental factors; and (3) the number of genes or gene blocks which control the factor studied. Lack of adequate dietary protein is a condition which affects a large proportion of the world population. Our program attempts to determine the effect of diet (such as different proportions of protein in the total diet), during particular stages of the life span upon behavior and longevity for animal populations which differ in genetic constitution.

Studies of single-gene mutant animals are of importance because they allow the assessment of the importance of a specific genetic locus for physiological or behavioral factors.

Proposed Course of Project: Further inbred strains and F_1 hybrid groups are being studied to determine the generality of mode of inheritance for behavioral factors. Cross-sectional and longitudinal studies of mouse behavior will continue with mouse strains BALB/cJ and DBA/2J, in addition to the A/J and C57BL/6J mouse strains. The longevity of inbred and hybrid groups are also being determined. Experiments with low and normal protein diets should determine: (1) the relationships between growth rate and longevity, (2) the effect of low, normal, or high protein diets upon behavior at maturity after access to these diets during various stages of development, and (3) the effects of a present diet of low, normal, or high protein upon behavior.

Honors and Awards: None

Publications: Goodrick, C. Exploration activity and emotionality of albino and pigmented mice: Inheritance and the effects of test illumination. Journal of Comparative and Physiological Psychology, In Press.

TABLE 1

Mean scores of free exploration as a function of group, diet, and number of mice per cage.

NUMBER PER CAGE	DIET	GROUP C57	F_1 (MP)	A/J
1	Low	39.8	28.3(20.1)*	0.3
	Normal	39.9	17.9(20.5)	1.0
2	Low	40.2	27.2(23.5)	6.8
	Normal	39.8	22.2(20.2)	6.0
5	Low	33.5	31.5(18.2)*	2.9
	Normal	40.9	17.3(20.7)	0.5

* $p < .05$, F_1 vs. MP

Serial No. HD-AG 27

1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Exercise, general activity level and aging

Previous Serial Number: Same

Principal Investigator: Charles L. Goodrick, Ph.D. (30%)

Other Investigator: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.60
Professional:	.60
Others:	.00

Project Description:

Objectives: The general objectives are: (1) to determine methods for increasing physical activity of lower animals, (2) to examine behavioral and longevity differences among animals which differ in physical activity level, and (3) to determine the physiological mechanisms underlying differences in activity.

Methods Employed: Wistar rats are placed in activity wheels and allowed limited access to food. The result is an increment in physical activity (running) compared with normally fed animals or control animals. Hungry animals may also be rewarded with food for running. Other studies utilize inbred, hybrid, and mutant mice or species which differ in activity level due to different genetic constitutions. (See project HD-LB 1 Behavioral genetics and aging).

Major Findings:

A. In the past the major research in this area in our laboratory was concerned with exploration behavior and variables which may act to reduce age differences in exploration. Having completed a series of experiments concerning exploration and aging, some of the present projects are directly examining the effects of exercise upon longevity and exploration behavior for mice and rats. At this time last year, the longevity data were incomplete

for an experiment in which the effects of access to an activity wheel for a 30-day period were determined for F_2 hybrid mice. Male and Female F_2 hybrid mice of black, brown, or white (albino) coat colors were studied. One-half of each of six groups (N=20/group) were allowed 30 days of free exercise at 23-months of age and the other half were placed in individual cages during this period. The previously reported data clearly found an increment in exploration activity for groups allowed wheel exercise. Additional analyses are now complete for measures of longevity and behavior as a function of coat color, sex, and exercise condition. Mean scores of longevity, wheel activity, exploration activity, and emotionality are given in Table 1 as a function of exercise condition and sex. The only main effect that was statistically significant for the longevity score was that of Condition, F (1,108)=8.07, $p < .01$. This effect must be considered in relation to the Condition x Sex interaction, F (1,108)= 8.24, $p < .01$. Although the differences were significant for male mice, t (28)= 2.56, $p < .05$, differences were not significant for female mice. Coat color did not affect longevity for this mouse population. Body weight for female mice was significantly lower than for male mice, F (1,108)= 4.05, $p < .05$, the only significant difference for this measure. Control and experimental mice had been paired on the basis of body weight prior to wheel tests. Significant differences in wheel activity were not obtained with respect to coat color or sex for these groups. Wheel activity averaged over 3,000 revolutions per day, much less than mean scores obtained for mature-young mice (5 mo. old) of 10,000 revolutions per day. Mice allowed access to the activity wheels engaged in significantly more exploration activity, F (1,108)= 18.14, $p < .01$, and were significantly less emotional, F (1,108)= 7.54, $p < .01$, than control mice (Table 1). Sex differences and condition differences were not obtained and interactions were not statistically significant for either measure.

B. Recent studies of immature nursing rat pups by other investigators have suggested that immature 16-18 day old pups are highly active in a novel environment. The interpretation of these data was that there is a peak in exploration activity at this age. Our previous research found that recently weaned rats 31 days old scored very high on tests of exploration, and after 3-6 months of age exploration decreased as a function of increasing age. A series of experiments were completed to determine the tendency of nursing rat pups to leave the familiar nest area, and additional data were obtained to determine the amount of exploration of a novel environment for rats 16, 24, and 32 days old. Rat pups (N=73) did not consistently leave the nest area to explore a novel environment until 23 days of age when tested at 17, 19, or 23 days of age, although parents explored at a high level during all three tests. Tests of forced exploration (dependent upon locomotor ability) and free exploration (independent of locomotor ability) indicated that exploration activity was a monotonic increasing function of age for rat pups age 16, 24, and 32 days old. These data were in agreement with previous work from our laboratory which found that immature rats 31 days old are at a maximal stage of exploration activity, compared with exploration activity of mature-old or aged rats.

C. An experiment has been completed to determine the adaptation to a novel environment during a single test of long duration by rats over the age range of 16 to 750 days of age. Groups included nursing rat pups 16-18 days old, immature weaned rats 24-32 days old, mature-young rats 150-180 days old, and aged rats 750-800 days old (N=96, 24/group). Rats were tested individually in 2-bar operant test chambers for 2-hour intervals, with the number of bar presses recorded every 15-minutes during the 2 hours. Preliminary analyses indicate that rats 16-18 days old do not adapt to the environment over a 2-hour test interval. Bar pressing responses remain at moderate levels throughout the entire test period. Immature, mature-young, and aged groups adapt quickly to the novel environment, with most responses in the initial 15-minutes of the 2-hour test interval. It was hypothesized that the failure to adapt to the novel environment by 16-18 day old nursing rat pups was due to strong motivation to return to the home nest, rather than to a strong exploration drive, as other experimenters had suggested. An additional experiment was completed to test the hypothesis that immature nursing rats 16-18 days old would adapt to the novel environment if group tested with their littermates. Moreover, it was expected that young immature rats 24-32 days old would adapt less quickly if tested in groups with their littermates. Rats at these two ages (16-18 days and 24-32 days were tested individually, in pairs, or in groups of three. The results were in complete agreement with the hypotheses. Groups 16-18 days old adapted more quickly to the novel environment as a function of increasing number of pups in the test environment. Rats 24-32 days old adapted less quickly to the novel environment as a function of increasing number of immature rats in the test environment.

Significance to Bio-Medical Research and the Program of the Institute: One of the consistent findings of gerontological research is the decline in general activity level of old animals compared with young animals. It is important to determine whether quantity of activity (e.g. wheel activity) and/or quality of activity (e.g. increased exploration behavior or greater response variability) may be increased for old and senile animals. It is also important to examine the role of heredity with respect to voluntary exercise throughout the entire lifespan, and the effect of exercise upon behavioral decrements associated with advanced old age.

Proposed Course of the Project: Mature-young, mature-old, and aged rats will be trained to increase base activity level for food rewards to determine: (1) maximum increases which may be obtained, (2) the durability of increases (i.e., if increments in activity are maintained after training ceases), and (3) if increased activity level results in transfer to other tests or conditions. Data will be analyzed to determine: (1) correlations between general activity level and longevity and (2) longevity for exercised and nonexercised groups of project A. Projects B, C, and D will be continued.

Honors and Awards: None

Publications: None

TABLE 1

Mean longevity, body weight, wheel activity, exploration, and emotionality for male and female mice as a function of exercise

	EXERCISE		CONTROL	
	MALE	FEMALE	MALE	FEMALE
Longevity	30.4	29.8	27.5	29.8
Body Weight	29.5	28.2	29.4	28.3
Wheel Activity/1000	32.5	33.8		
Exploration	50.9	49.7	37.0	37.2
Emotionality	7.0	6.6	9.9	9.7

Serial No. HD-AG 25

1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Section on Learning and Problem Solving
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Operant performance, memory and aging

Previous Serial Number: Same

Principal Investigator: Charles L. Goodrick, Ph.D. (30%)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.60
Professional:	.60
Others:	.00

Project Description:

Objectives: Research on both human and animal subjects has shown that short-term memory deficits and motor performance deficits are major problems of aged individuals. An interpretation of maze learning differences between young and aged rats is that these differences are due to short-term memory deficits of the old animals. The general objectives of one phase of this project are: (1) to analyze complex maze learning of young and aged animals; and (2) to determine variables which may act to enhance or retard maze learning ability. The objectives of the other phase of this project are: (1) to study age differences in motor performance during operant responding; and (2) to develop operant techniques which improve the retention of learned responses in aged animals.

Methods Employed: Operant conditioning performance and retention studies have used 2-bar test boxes in which hungry animals are trained to press one bar to obtain a food reward while the alternate bar remains neutral. By increasing the complexity of the task (using two bars rather than one), it is possible to make a finer analysis of performance and the retention process. We are studying performance and retention as a function of reward schedule, and we are particularly interested in the partial reinforcement effect. The retention of partially rewarded responses is vastly greater than for responses continuously rewarded; and analysis of this phenomenon will provide information regarding the general retention process. A complex 14-unit multiple-T maze is also utilized. This maze has been shown to have a high degree of reliability and has been used in many major studies of aging and learning. Additional

mazes of 6-units are being developed to study mastery of consecutive problems by young and aged rats. These mazes will be used to analyze aging effects in short-term and long-term memory, and determine aging effects in proactive and retroactive inhibition.

Major Findings:

A. Studies in the last few years from this laboratory have utilized a complex maze problem to show clear age differences in learning ability for mature-young and aged rats. One test factor found to reduce age differences was distribution of practice. Aged rats given massed practice were able to reduce error scores (a measure independent of activity level) to the same level as that of young rats. During distributed practice trials, aged rats make perseverative errors within the maze, i.e., they tend to repeat errors over trials at the same culs-de-sac to a much greater degree than young rats. Further information is now available with respect to changes in perseverative error frequencies and percentages over a long series of trials for young and aged rats tested with distributed trials or moderately massed trials. The analysis was a 2X2 design (Practice Condition X Trials), using the percentage scores of just the aged rats. The aged distributed practice groups had a significantly higher percentage of perseverative errors than the aged massed practice groups, F (1,30)= 10.04, $p < .01$. This was due to an increase in the percentage of such errors over trials for the aged distributed practice groups, while a decrease in the percentage of such errors occurred over Trials for the aged massed practice group, Practice Condition X Trials, F (1,30)= 12.24, $p < .01$. Although the group mean differences in the percentage of perseverative errors were not statistically significant on trials 1-20, group mean differences were highly statistically significant for Trials 21-40, t (30)= 6.75, $p < .01$. Table 1 gives the mean number of culs-de-sac where six or more errors occurred, mean number of perseverative errors, and mean percentage of perseverative errors as a function of practice condition and age. On the basis of past research in this laboratory it was hypothesized that age differences in learning were due to short term memory deficits of aged animals. An analysis of the elimination of perseverative errors during training of aged rats utilizing moderately massed practice (4 trials/day) has given additional support to this hypothesis. Errors at specific culs-de-sac were normally obtained on initial trials during the daily 4-trial sequence. Correct responses were obtained on the later trials during the daily 4-trial sequence, until mastery of the cul-de-sac occurred with a correct turn occurring during all future trials (Table 2). This finding, based on a detailed analysis of individual records of the exact path of aged rats over a long series of learning trials, provides strong evidence that learning deficits of aged animals may be the result of short-term memory deficits.

B. Another hypothesis, based on our earlier research, was that aged animals would benefit more from a forced correct sequence early in the training series than younger animals. Groups of mature-young and aged rats have been given initial tests in which barriers are placed at all culs-de-sac so that all animals must run the correct sequence of 14 turns after which they receive a food reward. After these preliminary tests, the rats were given additional learning trials with the barriers removed. During these learning trials the aged rats made fewer errors than the young rats and all

aged rats quickly mastered the maze problem. The implications of this finding are that learning may be facilitated more for slow learners than for fast learners through procedures which require active correct responses early in training. Further animals are to be tested and the results will be given in detail in the next annual report.

C. An important aspect of the animal behavior program has been the systematic investigation of the retention of learned responses following training utilizing operant test chambers. During these tests the influence of complex schedules of reward upon performance during training and extinction after training may be determined. The use of two-bar test boxes with one bar reward contingent and the other bar neutral allows an assessment of performance (number of responses) which is independent of the learning measure (percentage of reward bar responses), an important feature for studies which may involve active young animals and inactive aged animals. A further study in this series has examined operant performance for young and aged rats on fixed interval schedules of reward. This type of schedule is time-contingent, i.e., a reward is given for a response following a fixed time interval, but the number of responses is unspecified. The major findings of this experiment were that during training number of responses per reward was in increasing function of the duration of the fixed interval, but number of responses per reward did not differ significantly for motivated mature-young and aged rats. This experiment provides further support for results obtained earlier in our laboratory that number of responses during operant testing failed to yield an expected result of lower levels of response for aged than young animals. Furthermore, the total level of responses was similar for young and aged rats during extinction trials, and the percentage of reward bar responses during training and extinction trials were also similar for young and aged rats. The importance of this essentially negative finding is being examined in relation to the very large age differences obtained for other tests with a high activity component such as wheel activity or free exploration activity.

Significance to Bio-Medical Research and the Program of the Institute: Learning and/or memory deficits represent a major problem among the aged human population. Major behavioral or physiological techniques to reduce performance deficits obtained for aged, lower animals have been studied in our laboratory. This project may facilitate research with higher organisms such as man by determining the optimal conditions for learning and for retention of learned responses.

Proposed Course of Project: Further studies are in progress to determine the nature of the partial reinforcement effect in relation to (a) time contingent vs. response contingent partial reinforcement and, (b) massed vs. distributed extinction trials. Other studies will examine age differences in operant performance as a function of response effortfulness. Maze studies will concentrate upon the effects of central nervous system stimulants on behavioral rigidity within the maze for old rats. We will also initiate preliminary studies of rote learning and perceptual learning of human subjects.

Honors and Awards: None

Publications: Goodrick, C. Learning by mature-young and aged Wistar albino rats as a function of test complexity. Journal of Gerontology, 1972, 27, 353-357.

Goodrick, C. Error goal-gradients of mature-young and aged rats during training in a 14-unit spacial maze. Psychological Reports, 1973, 32, 359-362.

Goodrick, C. Maze learning of mature-young and aged rats as a function of distribution of practice. Journal of Experimental Psychology, In press.

TABLE 1

Mean number of culs-de-sac where six or more errors occurred (a), mean number of perseverative errors (b), and mean percentage of perseverative errors (c) as a function of practice condition and age.

Practice Condition	Group	Trials 1-20			Trials 21-40		
		a	b	c	a	b	c
Distributed	Young	5.4	0.9	14.3	0.4	0.0	0.0
Massed	Young	6.5	1.4	19.8	0.4	0.0	0.0
Distributed	Aged	7.1	3.8	50.5	3.8	2.5	64.9
Massed	Aged	6.9	3.4	49.1	2.9	1.3	34.2

TABLE 2

Errors at thirty-three perseverative choice points as a function of series and trials for aged rats given massed practice

Trials	Series						
	A	B	C	D	E	F	G
1	33	31	25	18	7	5	0
2	33	23	13	10	5	1	0
3	33	17	12	5	0	1	0
4	33	5	4	2	0	0	0
	33	33	26	21	8	5	0
	Total Perseverative Choice Points						

Serial No. HD-AG16 (8) (c)
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Learned Modification of Visceral Function in Man

Principal Investigator: Bernard T. Engel, Ph.D. (40%)

Other Investigators: Donald A. Kristt, M.D. (45%)
Marvin G. Schuster, M.D. (5%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	2.20
Professional:	.90
Other:	1.30

Project Description:

Objectives:

A. Learned control of blood pressure.

1. To determine whether patients with essential hypertension can be taught to control their systolic blood pressure (SBP) through operant conditioning techniques.

2. To determine whether patients so trained can achieve a significant and sustained lowering of their abnormally elevated blood pressure.

B. Learned control of anal sphincter function.

1. To determine whether patients with severe, intractable fecal incontinence (continuous daily soiling) can be trained to control anal sphincter responses.

2. To determine whether responses of the internal as well as the external anal sphincter can be brought under voluntary control.

3. To determine whether such control, once learned, will lead to clinically significant improvements in lower bowel function.

Methods Employed:

A. Learned control of blood pressure.

Volunteer patients are recruited from the Baltimore City Hospitals (BCH) Hypertension Clinic. During the study patients are hospitalized from one to three weeks on the research ward of BCH. Patients are trained in the laboratory to raise and lower median SBP. During a typical experimental session the patient lies semireclined in a hospital bed. Arranged in front of him are a vertical display of three lights. The top light is green, the middle light is yellow and the bottom light is red. In addition, there is a digital percent meter that provided the patient with a cumulative record of his success during a trial. A standard blood pressure cuff, covering a small crystal microphone, is applied over the ante-cubital fossa. The microphone detects the presence of Korotkoff's sounds.

Each session is divided into three phases. Phase 1 is a determination of baseline SBP. Before going on to phase 2, the patient's blood pressure has to become stable over at least five trials. Each trial is 50 heart beats in duration. Following the 50 beat trial the BP cuff is deflated and the patient is permitted to rest for 32 sec. before the next trial.

Phase 2 consists of a battery of 18-24 training trials punctuated by several short pauses. During this phase the patient tries to control his blood pressure. Phase 3 is a post-training SBP baseline of 5 trials.

If the patient is being trained to raise his blood pressure, the green light is turned on and remains lit throughout the training trial. Lights are off during baseline phases, and inter-trial rest periods. When the patient raises his SBP (i.e., produces more Korotkoff sounds (KS) at a fixed cuff pressure in a given trial), the yellow light goes on and remains on as long as Korotkoff sounds are produced. If the patient's SBP fell below cuff pressure for a given heart beat and no KS was produced, then the yellow light would go off. Each heart beat accompanied by a KS during "raising" training registers + 2% on the cumulative percent meter. Each patient is instructed to keep the yellow light on as much as possible and to accumulate as high a percent score as possible.

If the patient is being trained to lower his SBP below cuff pressure, reinforcement (the yellow light) is provided when production of KS is suppressed. In this case the percent meter reflects the number of heart beats not accompanied by KS.

After the patient has been trained separately to raise and to lower his SBP, he is further tested under differential training conditions. During the training phases of these sessions the patient is required to alternately raise and lower his SBP over 6-8 consecutive trials. The onset of each training condition is signaled by the appropriate cue lights, either green or red.

B. Learned Control of anal sphincter function.

Sphincteric activity is monitored by means of pressure recordings from a triple balloon system. One balloon is placed in the rectum, a second balloon is placed at the internal sphincter and the third balloon is placed at the external sphincter. In normal subjects distension of the rectum elicits reflex relaxation of the internal sphincter, and simultaneous contraction of the external sphincter.

Each patient in this study was evaluated in three ways: 1) his clinical history was taken; 2) rectal examination was conducted to determine clinically the degree of sphincteric tone; 3) the rectal distension procedure described above was employed: a) to determine which sphincter was malfunctioning, and b) to determine the threshold of response of the sphincteric reflex. Thresholds were determined by systematically inflating the rectal balloon with a known volume of air and simultaneously observing the sphincteric responses.

Once the patient's threshold response was determined, a series of baseline recordings was made to provide an objective basis for measuring change. After the baseline measurements were made, the patient was permitted to observe the tracings from the pressure manometer, and he was instructed to "make the responses look like normal ones." The patient had received instructions earlier on the nature of normal sphincteric activity. Depending upon the particular abnormality the patient manifested, he would be instructed to try to control either sphincter or the synchrony between the sphincters.

Patients were seen in one to three training sessions separated by periods ranging from two to six weeks. Improvement was measured in terms of change in sphincteric activity in the laboratory, in terms of the physicians judgement of the change in anal sphincteric tone and in terms of the patient's report of his ability to remain continent.

Seven patients were seen. Two were men and five were women. Age range was 6 years to 55 years. The patients had histories of fecal incontinence ranging from one year to several years. In three of the patients the tests indicated abnormal motor responses in both sphincters and in the other four patients only the external sphincter malfunctioned.

Major Findings:

A. Learned control of blood pressure.

One patient completed three weeks of conditioning. She is 60 years old and has mild essential hypertension of at least 25 years duration. Her hypertension showed marked exacerbation during her only pregnancy in 1954. SBP at this time was 260 mm Hg. Presently she is mildly hypertensive, well controlled on diuretic therapy. Her mean SBP during clinic visits over the past year was 147.5 mm Hg (n = 15 visits). During the period of her training while she was hospitalized, diuretic therapy was discontinued and low salt diet instituted in its place. This was done to obviate the common hypotensive side effects of hospitalization

of active, medically controlled hypertensives.

This patient was hospitalized for three weeks. She was first trained to raise, then to lower and finally to alternate raising and lowering of SBP.

The first stage of training consisted of nine raising sessions. The mean elevation over baseline SBP for these sessions was 10 mm Hg. In one session, toward the end of the block of "raising" sessions, an elevation of 20 mm Hg. was achieved.

The initial and terminal baselines, phase 1 and phase 3, were generally quite similar differing by a mean of +0.7 mm Hg. in all but one session in which the terminal baseline rose 13 mm Hg. Cuff pressure was adjusted on a trial to trial basis so that 25-75% of Korotkoff's sounds were produced each trial: i.e., cuff pressure was adjusted to match median SBP throughout the session.

The results during the subsequent 14 lowering sessions are less clearcut. In seven sessions lowering of SBP ranged from 4-19 mm Hg. (mean 8.6 mm Hg.) below initial baseline. In the remaining seven sessions mean lowering was 0.7 mm Hg. However, the terminal baseline was raised over both initial baseline and training level. Mean baseline differences in these latter seven sessions was +5.0 mm Hg. as compared to the mean difference in the successful sessions of -1.2 mm Hg. This suggests that although lowering did not occur in these sessions elevations of SBP during training were suppressed. No trend relating level of initial SBP and degree of lowering was apparent.

The final stage of training was 12 alternation sessions. In the same session the patient was asked to first lower SBP for six trials then raise it for six trials and finally to lower from this new level for a final eight trials. The patient had most difficulty lowering after raising SBP. The mean initial lowering was -4.8 mm Hg.; mean raising +6.1 mm Hg. During final lowering SBP rose by a mean of 1.7 mm Hg. In the last four alternate sessions, terminal lowering was achieved: mean -2.3 mm Hg. In the sessions prior to discharge, the patient's alternate record was (L)-4, (R)+8, (L)-14 mm Hg.

The patient returned one month following training for a follow-up alternate session. The patient was medicated in her usual fashion during this session. Her alternate record at this time was (L)-5, (R)+7, (L)-4 mm Hg.

In addition to the laboratory studies this patient also was taught to take home blood pressures. The results from this phase of the study show a mean systolic BP of 127 mm Hg. (n=525 determinations over three weeks) as compared to clinic SBP of 147.5 mm Hg. during the previous year; the patient also is able to voluntarily lower her SBP from this average level by a mean of 17.6 mm Hg. using this device (this mean also was based on 25 readings/day for three weeks).

B. Learned control of anal sphincter function.

One of the patients who had a history of an anal fissure withdrew from the study after one session because the measurement procedure was painful. None of the other patients complained of pain. Five of the six patients have shown significant improvement (defined as no soiling according to patient's self report), which has persisted since the time of study. The periods of improvement are: 2 mos., 6 mos., 1 yr., 2 yrs. and 3 yrs. One patient reported some improvement (defined as reduced soiling according to patient's report). He has sustained his control for about 1 yr. to date.

In addition to the changes in continence reported by the patients, there are objective signs of significant improvements in bowel function. Tracings taken from each patient while he was in the laboratory show systematic signs of improvement. This effect is true for the internal sphincter responses as well as for responses of the external sphincters.

In addition to the scientific implications of these results, the clinical effects are especially interesting. The procedure utilized was adapted from the diagnostic procedures now used by many gastroenterologists. Thus, the technology for applying these techniques clinically is already available to practicing physicians. A second factor which adds to the clinical potential of this conditioning procedure is that the patients were able to learn to control their sphincters quickly. In no case was more than three sessions necessary.

Significance: This project is designed to increase our understanding of learned control of autonomic responses and the application of this knowledge to the control of various illnesses. These data add further credence to the conclusion that responses mediated by the autonomic nervous system can be brought under voluntary control, and they show that such control holds out the possibility of clinically useful applications.

Proposed Course: At least five additional patients with high blood pressure will be studied. If these techniques prove efficacious with these patients, they may be extended to evaluate the clinical efficacy of blood pressure control in the regulation of pain associated with angina pectoris.

Honors and Awards: Dr. Engel presented a lecture (by invitation) to the joint meeting of the Maryland Psychiatric Association and the Maryland Psychological Association (December, 1972)

Dr. Engel presented at the medical grand rounds of the Rhode Island Hospital (March, 1973)

Dr. Engel presented at the cardiology grand rounds of the Johns Hopkins Hospital (March, 1973)

Publications: Bleecker, E.R. and Engel, B.T. Learned control of ventricular rate in patients with atrial fibrillation. Psychosomatic Medicine, 1973, 35, 161-175

Bleecker, E.R. and Engel, B.T. Learned control of cardiac rate and cardiac conduction in the Wolff - Parkinson - White Syndrome. New England Journal of Medicine, 1973, 288, 560-562.

Engel, B.T. Operant conditioning of cardiac function: Some implications for psychosomatic medicine. Behavior Therapy, in press.

Engel, B.T. Clinical applications of operant conditioning techniques in the control of the cardia arrhythmias. Seminars in Psychiatry, in press.

Serial No. HD-AG2 (8)
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Learned Modification of Visceral Function in Animals

Previous Serial Number: Same

Principal Investigator: Bernard T. Engel, Ph.D. (55%)

Other Investigators: George W. Ainslie, Jr., M.D. (90%)
Donald A. Kristt, M.D. (45%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	4.20
Professional:	1.80
Other:	2.30

Project Description:

Objectives:

A. Operant Conditioning of Heart Rate.
To train monkeys to voluntarily control their cardiac functions, and to use this preparation to identify the physiological and psychological mechanisms associated with this control.

B. Depressor activity of the rat hypothalamus.

1. To locate and define vasodepressor areas in the rat diencephalon.

2. To develop a chronic, awake preparation in the rat in which such an effect can be elicited and continuously monitored.

3. To utilize this hypotensive effect to determine whether spontaneously hypertensive rats would engage in intracranial self-stimulation to obtain this transient lowering of blood pressure.

Methods Employed:

A. Operant Conditioning of Heart Rate.
Three studies have been performed during the last year:

1. Interaction of operant and respondent conditioning of cardiac rate.

Two factor learning theory has long held that operant learning is greatly affected by respondent conditioning to cues in the experimental environment, however, the possibility that respondent conditioning can be altered by operant incentives has received little attention. This has probably been so because operant learning was believed until recently to take place only with skeletal muscle responses. Since evidence for respondent conditioning of these responses has been equivocal, there has been no experimental preparation in which operant modification of respondent conditioning could be studied. The recent discovery that visceral responses can be operantly conditioned makes such an experiment possible.

Subjects were six monkeys of which five were naive and one had previously had extensive training in cardiac slowing as an operant to avoid electric shock. Each subject had its external iliac artery cannulated under sterile surgical conditions. The polyethylene catheters were kept open by continuous infusion of heparinized normal saline at the rate of about 20 units/hr. After surgery each subject lived in its own restraining chair in an individual booth which was sound-deadened when the door was closed.

Each animal usually received four experimental sessions each weekday. The subject's door was closed and no stimuli were introduced for 20 min. to allow its heart rate and blood pressure to reach a stable level. These responses were then measured for 512 sec. by an on-line Raytheon 704 computer, which then conducted a 2048 sec. experimental session. The sessions were one of three kinds: Operant rate control to avoid electric shock, respondent conditioning to a stimulus predicting an unavoidable electric shock, or a combination of both.

Operant rate control was taught by means of feedback from a row of three lights facing the subject. Throughout each session one of the side lights was on, warning the subject that it must speed or slow its heart or receive a 0.5 sec, 10 ma shock to its tail. A green light on the subject's left commanded speeding, and a red light on its right slowing. The white light in the center was on whenever the last inter-beat interval had met a criterion based on baseline heart rate. A free running clock produced an electrical impulse every 8 sec., and if the white light was off when this impulse occurred the monkey received a shock. It was safe from shock whenever the white light was on, signalling that its rate was meeting the criterion.

During respondent conditioning the cue lights were never on, but 128 sec. periods of clicking from a loudspeaker alternated with 128 sec. period of silence. The frequency of the click alternated in turn between two and 20 clicks/sec., so the following pattern repeated itself every 512 sec: 128 sec. of 2/sec. clicks, 128 sec. of silence, 128 sec. of 20/sec. clicks, 128 sec. silence. For each monkey one of the click patterns (warning clicks) was always followed by four rapid 0.1 sec., 10 ma shocks to the tail; the other click pattern (neutral clicks) was never followed by shock.

During the combined condition, both of the above schedules were in force simultaneously.

Two subjects were initially conditioned to the clicks. When their cardiac responses to the clicks were stable, they were given rate control sessions in which they learned to slow their heart rate to avoid shock. Two other subjects including the experienced monkey began with rate control training. After each subject had learned to change its rate in the desired direction, it was given more respondent conditioning until its cardiac responses were again stable. It was then put in the combined situation, and then in the respondent-only situation again. This process was repeated with rate training in the opposite direction for two animals. In addition to these subjects two animals which were trained to speed their hearts first also are in this group.

2. The effect of chronic, sustained, operantly conditioned cardiac changes on cardiovascular function.

The purpose of this project is to learn whether monkeys can be made to develop abnormal cardiac responses through conditioning procedures. Two animals are now being studied. Each animal participates in a three-phase study. Phase 1 is a four week baseline period during which each animal's heart rate and blood pressure is continuously monitored for 18 hr/day, five days/wk. The monitoring period is from 6:00 PM until noon the following day. Phase 2 is a three week period during which each animal is taught to control its heart rate to avoid an electric shock. Animal 1 was trained to speed its rate and animal 2 was trained to slow its rate.

Phase 3 is of indeterminate length, however, it will persist for at least 6 mos. During phase 3 each animal is in a training situation 18 hr/day, five days/wk (from 6:00 PM until noon). As long as the animal maintains its heart rate above (animal 1) or below (animal 2) a criterion rate, the animal is "safe." Whenever its rate is outside of the criterion region, the animal accumulates "bad time." When the animal accumulates 64 sec. of bad time, the appropriate shock-avoidance contingency is turned on and remains on for 1024 sec. (about 17 min.). Thus, the animal must maintain a certain level of heart rate to avoid turning on its schedule, and it must avoid shock whenever the schedule is on.

The criterion heart rate which determines the schedule is set by the experimenter, and it is made progressively more difficult as the study proceeds. Animal 1 which has been in phase 3 for two weeks at this time, and which must maintain a relative tachycardia, had its criterion set at 100 beats/min. initially, and now has a criterion of 120 beats/min. Animal 2 which has been studied for about six weeks now, and which has to maintain a relative bradycardia, had its criterion set at 200 beats/min. initially, and now has its criterion set at 160 beats/min.

B. Depressor Activity of the Rat Hypothalamus.

Twenty-two male Wistar rats were utilized. 13 were 6 to 11 mos. of age; the other 9 were 5 mos. of age. All rats were anesthetized with pentobarbital, 40-59 mg/kg. administered intraperitoneally. During the deep phases of anesthesia an arterial catheter (either femoral or carotid) was placed and the animal was prepared and fixed in a sterotaxic apparatus. Blood pressure and heart rate were continuously monitored throughout the experiment. While the rat was in the stereotax, an electrode was placed unilaterally so that its exposed tip was in the region of the antero-lateral hypothalamus medial to median forebrain bundle. A zone 1.2 mm. lateral to the midline was explored from approx A. 2.0 to A 6.2, H 6.0 to 9.2 (Massopust, L.C. Jr., Diencephalon of the rat (in) Sheer, O.E., Electrical stimulation of the brain., 1961, Univ. of Texas, 182-202). Exploration was first performed with monopolar electrodes since it seemed likely that vasodepresser effects might involve several structures. Stimulation consisted of trains of square-wave pulses. They were produced by a Grass square wave generator via an isolated constant current unit. Stimulation parameters were as follows: Train duration (TD) 2500-5000 ms; Train frequency (TPS), 0.1 Trains/sec.; pulse duration (PD), 1-8 ms; pulse frequency (PPS), 50-200/sec. Current levels ranged from 100 to 750 ua.

For refinement of the localization and elucidation of the mechanism of action of the stimulation a concentric bipolar electrode was utilized. Stimulation paremeters were: TD, 50-5000 ms.; TPS, 0.1-1.0; PD, 5-8 ms; PPS, 100. Current levels ranged from 50-400 ua. Two vasoactive drugs were employed to produce changes in blood pressure levels. Isoproterenol and epinephrine were used in doses of 1-4 µg/kg. body weight.

All rats were decapitated and their brains fixed in 10% formalin for gross and/or microscopic evaluation of electrode localization.

Major Findings:

A. Operant Conditioning of Heart Rate.

1. Interaction of operant and respondent conditioning of heart rate.

It often required several weeks for the animals' behavior to stabilize. Because of this, only the first half of the experiment has been completed and analyzed on the first four monkeys, despite the fact that they have been tested as long as ten months. This study will be completed by the end of this reporting period, however. The initial rate training for all four was in slowing, and clear data exist about this manipulation. The two monkeys which began with respondent conditioning (20 sessions) quickly learned to raise their heart rates significantly during the warning click (6 and 37 beats/min. during the last week of conditioning, t=3.29 and 9.07 respectively). During rate training (about 30 sessions for the previously naive subjects) all four monkeys learned to slow their rates significantly. The average change in rate for all four was 14 beats/min. Three of them rarely received avoidable shocks after their initial training, but one had intermittent episodes of poor performance and high rate of shock. During subsequent respondent conditioning trials (30-90 sessions), all four speeded in the presence of warning clicks. The average heart rate during warning

clicks for all four animals was 5 beats/min higher than during neutral clicks, and 12.3 beats/min higher than in the absence of auditory stimuli.

When operant and respondent schedules were in force simultaneously (80-130 sessions), three of the four monkeys developed lower rates during the warning than during the neutral clicks; the exception was the monkey whose rate control was imperfect, and even it showed less tendency than it had previously to speed during the warning clicks. The subjects' mean rate during warning clicks was 7.0 beats/min lower than during actual clicks, 13.4 beats/min lower than in the absence of auditory stimuli. These changes in the subjects' conditioned heart rates persisted after they returned to the respondent-only schedule. Although one monkey reverted to its original pattern of differential speeding after 40 sessions, the change of direction in the others was stable throughout more than 80 sessions. Systolic and diastolic blood pressure during rate training differed from subject to subject, however, they were generally consistent over time for each individual. During respondent conditioning blood pressures rose during the warning clicks for all subjects throughout the experiment regardless of the direction of rate change, and changed virtually not at all during the neutral clicks.

Table 1 summarizes the data from all phases of this study.

TABLE 1

Average Changes in heart rates (beats/min) and blood pressures (mm. Hg.) during different phases of the study (Cond 1, respondent conditioning before pairing; Comb, simultaneous respondent and operant conditioning; Cond 2, respondent conditioning after pairing).

	Cond 1	Comb	Cond 2
Heart Rate			
Warning clicks	5.9	-18.7	-4.7
Neutral clicks	0.9	-11.7	-2.3
No Clicks	-6.4	-15.3	-5.7
Systolic Pressure			
Warning clicks	8.8	4.2	4.0
Neutral clicks	-1.8	-0.4	-2.6
No clicks	-1.9	-0.1	-0.4
Diastolic Pressure			
Warning clicks	6.9	2.2	2.7
Neutral clicks	-0.7	-0.7	-1.7
No clicks	-1.2	-0.6	-0.2

2. The effect of chronic, sustained, operantly conditioned cardiac changes on cardiovascular function.

It is too soon to carry out a systematic analysis of the data in terms of such factors as diurnal variation, however, some general findings can now be reported. Animal 1 which was trained to speed its heart rate had average weekly heart rates before speeding training of 130, 137, 118 and 132 beats/min. Blood pressures were 130/77, 123/67, 120/65 and 118/64 mm Hg. During two weeks of post-training study its heart rates were 145 and 143 beats/min and its blood pressures were 132/78 and 136/83 mm Hg. Thus, under conditions of 18 hr. avoidance training it has been possible to maintain a relative tachycardia over two weeks. Obviously, it is much too soon to speculate beyond these data at this time.

Animal 2 was trained to slow its rate. Its average weekly rates before training were 147, 146, 146 and 140 beats/min. Blood pressures over this period were 146/107, 140/100, 125/82 and 118/76 mm Hg. After slowing training and during six weeks of continued avoidance conditioning its weekly average rates were 148, 152, 152, 139, 149 and 133 beats/min. Blood pressures were 124/80, 126/80, 117/74, 113/73, 120/78 and 111/72 mm Hg. Thus it appears that heart rate is beginning to fall below baseline levels while blood pressure remains stable.

During periods when the shock schedules are operative animal 1's heart rate is about 15 beats/min faster than when it is off schedule. Animal 2's heart rate is about 15 beats/min. slower when it is on schedule. These data are evidence that the schedules are still effective, and that the animals are still actively avoiding shock by selectively modifying their heart rates appropriately.

3. Autopsy findings.

Four monkeys that had had indwelling distal abdominal aortic catheters were autopsied. The catheters were in place for 3 mos. to 18 mos. In the two monkeys with catheters present for approximately 3 mos., moderately advanced arteriosclerotic changes were apparent. In one case, the catheter had been completely walled off by a fibrous intimal lesion. Adventitial fibrosis was conspicuous and lymphadenopathy was present locally. In a second case, thinning and mild aneurysmal dilation of the wall was noted in one area. The adventitial fibrosis and lymphadenopathy were similar. The remaining two monkeys showed striking arteriosclerotic aneursymal changes of the distal abdominal aorta. The longest lived catheter, 18 mos, was found in a thin walled, blood filled, friable aneursymal sac. The sac measured 14 cm in length and 6 cm in width. It was located in the left, lower retroperitoneum and was adherent to the subjacent flank muscles. The findings in these four animals suggest that the presence of continual intimal irritation from an indwelling catheter recapitulates the usual arteriosclerotic changes that eventuate in aneursym formation.

Two monkeys that had had a periaortic flow probe implanted were euthenized and autopsied after 3 mos. A loose fitting probe was used in one monkey. Fifteen percent of its inner surface perimeter was covered with dacron mesh. Histologic examination of the subjacent aorta showed a generally intact wall where peri-adventitial fibrosis involved the dacron mesh. The opposite wall (subjacent to bare probe) evidenced intra-medial hemorrhage and fibrosis with extensive loss and fragmentation of elastic tissue. In the second monkey the flow probe was 50% dacron covered and was a tighter fit. Here, there were no focal medial degenerative changes. However, there was a uniform band of sub-adventitial fibrosis in both the dacron protected and non-protected areas. This suggests that greatest longevity is most likely to ensue from a dacron wrapped, loosely fitting probe. The prognosis must be guarded for periods greater than 3 months using any other procedure.

B. Depressor Activity of the rat hypothalamus

1. Monopolar electrodes: A3.6, H7.7, L1.2 is a site where maximum monophasic depressor activity is produced: hereafter called SME. Generally, the effect is stable and reproducable with a mean fall in blood pressure of 14.7/14.5 mm Hg. (N=6). Occasionally pressures will fall 30 to 40 mm Hg., and sometimes a considerably smaller response is noted. Heart rates rarely changed during stimulation. Activity mapping in the L 1.2 plane showed that 1 mm anterior to the SME there was a silent zone. Further rostrad (A6.2), in the vicinity of the anterior commissure, vasodepressor activity was again observed. This is consistent with observations in the dog, cat and monkey. The effect was also noted caudal to the original site at A 3.2 (i.e., 0.4 mm posterior to SME) but disappeared by A 2.5 (1.1 mm posterior to SME). Activity in the horizontal plane was also explored. From the cortex to H 6.7 no change was noted. A minimal change was noted at H 7.2 of -6/-4 mm Hg. Moving down past SME the response becomes biphasic. The first phase is a brief response lasting approximately 500 ms or less. It is followed by a more prolonged depressor phase with a return to baseline in 3-7 min. The more ventral the stimulation the more conspicuous the positive phase. At the lowest point explored (H 9.2) the mean change in pressure for 2 consecutive stimulations for each phase was as follows 14/11, -16/-13 mm Hg. where the signs indicate pressor and depressor effects respectively. An analysis of stimulation parameters indicated that the depressor effect was a function of TD, PPS, PD and current. In general, decreasing the current density produces a smaller effect on pressure, however, this effect is not linear. Halving the current intensity produced a decrement of 1/3; lowering the current 4/5 halved the effect.

Histologic examination of the brains showed the tip site to be bounded by lateral hypothalamic neurons, the fornix and the cerebral peduncle. This agrees well with expected electrode placement. This raises 3 major options for mediation of the effect: (1) Direct stimulation or inhibitions of vasoregulatory neurons in the hypothalamus; (2) Indirect effect of skeletal muscle circulatory dynamics via the cerebral peduncle: (3) Indirect effect on the limbic system via the fornix.

2. Bipolar electrodes: Although evaluation of the data for this aspect of the work has not been completed, certain preliminary findings can be presented.

The depressor effect and its dependence on current density recapitulates the trend noted above. However, much lower current densities were needed: e.g., TD 500 ms, TPS 1.0, PPS 100, PD 8 and current 100 ua. The early positive phase, though usually small, i.e. 2-4 mm Hg. was more constantly present. The depressor phase showed considerable variablity: i.e., from 0 to 48 mm Hg. The variability was particularly interesting in that changes in the depressor phase were reciprocal with the pressor phase changes. That is, in the 5 rats evaluated to date in this regard, there were 4 episodes where the depressor phase was near zero. The pressor phase in three of these instances was a mean rise of 21.3 mm Hg. in diastolic pressure. Further evaluation revealed that the depressor effect was directly related to the instantaneous pre-stimulation pressure. The amplitude of the pressor phase was generally inversely proportional to the instantaneous pre-stimulation blood pressure, however, this latter relationship was less dependable than that noted for the depressor effect.

Preliminary evaluation of the effects of isoproterenol and epinephrine on the production of these changes shows: (a) that the direction and magnitude of stimulation induced change in blood pressure was inversely related to the net direction of change in the several minutes before stimulation. For example, during the recovery phase from epinephrine stimulation a pressor effect is produced. The depressor effect is zero despite the markedly elevated pre-stimulation pressure levels. (b) that despite the unchanging heart rate during usual stimulation, cardiac autonomic efferent nerves were also stimulated since stimulation plus isoproterenol produced a sustained cardiac arrhythmia. Isoproterenol alone did not produce an arrhythmia.

Significance to Biomedical Research and the Program of the Institute:

A. Operant Conditioning of Heart Rate.

Experiment 1 provides clear evidence that the form of at least one conditioned response, always regarded in the past as innate, can be modified by operant learning. This modification may persist long after it ceases to be differentially rewarded. While nothing can be said as yet about the mechanism or generality of this phenomenon, it offers an experimental approach to the large set of psychosomatic diseases that are apparently mediated by maladaptive autonomic responses. Experiment 2 also is important to our understanding of the nature of psychosomatic disorders. Despite the plethora of theoretical articles on the role of behavioral stress in the etiology of such illnesses, definitive experimental evidence of this process does not exist.

B. Depressor activity of the rat hypothalamus.

The work performed to date can provide a basis for further exploration of hypothalamic cardiovascular regulation. The rat appears to offer the advantage

that the spectrum of regulatory maneuvers are much more limited than those of higher mammals.

Propose Course of Project.

A. Operant Conditioning of Heart Rate.

In Experiment 1 comparison of the results of interpolated slowing training with results of speeding training should discriminate between the two most likely mechanisms for the operant modification of these conditioned responses: "Superstitious" failure of an avoidance response to extinguish (analogous to the self-punishment that has been described for some skeletal muscle operants), or some intrinsic effect of voluntary slowing that mitigates the aversiveness of shock. Trials using other unconditioned stimuli (air puffs, frights, etc.,) should provide data about whether the new responses generalize to other situations. This project will be completed within this fiscal year. As already indicated, experiment 2 will continue for at least 6 mos.

B. Depressor Activity of the Rat Hypothalamus.

Once the depressor site in the hypothalamus has been specified, phase 2 of the study will begin. In this phase rats will have chronically implanted electrodes in their hypothalamuses, and after they have been made hypertensive, they will be allowed to stimulate their brains electrically. Control animals which are normotensive also will be tested.

Honors and Awards: Dr. Engel lectured to the staff and fellows of the Center for the Study of Aging and Human Development, Duke University School of Medicine (February, 1973).

Dr. Engel presented a lecture at the Washington School of Psychiatry (March, 1973)

Publications: Engel, B.T. and Bleecker, E.R. Application of Operant Conditioning Techniques to the Control of the Cardia Arrhythmias. (in) Obrist, P.A., et al. Contemporary Trends in Cardiovascular Psychophysiology. Aldine-Atherton, Chicago, in press.

Serial No. HD-AG14 (8) (c)
1. Gerontology Research Center
2. Laboratory of Behavioral Sciences
3. Physiological Psychology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project title: Reaction Time and Age

Previous Serial Number: Same

Principal Investigator: Bernard T. Engel, Ph.D. (5%)

Other Investigators: Phillip R. Thorne (5%)

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.50
Professional:	.10
Other:	.40

Project Description:

Objectives: The purpose of this project is to study the effect of delay time on human reaction time, and to evaluate the function of age on this effect.

Method Employed: In the reaction time procedure used in this project, a subject is presented with an auditory signal (a tone to which he is supposed to depress a thumb switch as quickly as possible). The time between the onset of the tone and the switch press is the measure of his reaction time. Since the purpose of this project is to study the effect of time delay on reaction time, two time periods preceding each tone are systematically varied: 1) The time immediately preceding the onset of the tone--the foreperiod--is signalled by a light which goes on either 3 sec., 6 sec. or 12 sec. before the tone, and which remains on until the tone sounds; 2) The time between the end of one trial and the onset of the light of the next trial--the intertrial interval--is also either 3 sec., 6 sec. or 12 sec. By means of an appropriate statistical design, the effects of each of these time periods on reaction time, and the effect of the interaction between them on reaction time can be systematically studied.

Major Findings: Since this study has just begun, no results can be reported now. It is likely that data collection will be completed within one month, however.

Significance: It is well-known from a number of previous studies in this laboratory and elsewhere that older men have more difficulty coping with time pressure than younger men. This fact has been shown in studies of learning, problem solving, motor ability, and in intelligence tests. However, the mechanism underlying the aging effect is not known. The purpose of this project is to determine whether there are age differences in reaction time as a function of foreperiod duration and/or intertrial interval. If so, then it will be possible to utilize this model system to analyze the mechanisms which mediate the aging effect.

Proposed course: Two groups of 20 men each, one young (age 20-30) and one old (50-60) will be studied. Depending upon the results, a third, older group may also be studied. If an age effect is observed it will be possible, because of the experimental design chosen, to determine whether the effect occurs during the foreperiod or during the intertrial interval. Subsequent studies will then be designed to analyze the effect further.

Honors and Awards: None.

Publications: Engel, B.T., Thorne, P.R. and Quilter, R.E. On the relationships among sex, age, response mode, cardiac cycle phase, breathing cycle phases, and simple reaction time. Journal of Gerontology. 1972, 27, 456-460.

NICHD Annual Report
July 1, 1972 through June 30, 1973
Laboratory of Behavioral Sciences
Contract and Collaborative Research

Contract Number: NIH 70-2296
Contract Title: Twenty-five year follow-up analysis among former Army Air Force cadets.
Contractor: University of California (Los Angeles)
Money Allocated: None for FY 73
$10,673 (supplement for FY 72
36,251 (total for FY 71 and FY 72)

Objectives: In 1944 Dr. M. A. Wenger carried out a series of psychological and physiological tests on a group of 2111 Army Air Force cadets. In 1964 he sent health questionnaires to 1032 of these men and received 725 responses. On the basis of an analysis of these results it appeared that the 1944 tests might predict the occurence of some diseases among these men.

The purpose of this contract is to enable Wenger to carry out a survey of as many of the 2111 men as he can locate to determine the value of the 1944 test battery in predicting the subsequent development of certain psychological and physiological disorders. The reason this contract was initiated through this laboratory is that if the Wenger test battery does have predictive validity we would propose to include it in the longitudinal study program of the Gerontology Research Center.

Major Findings: It was possible to locate 1624 of the original sample of 2111 men (77%). Of these, 1444 (89%) were living and 180 were dead. Among the sample of 1624 men, 1248 who were physically fit at the time of testing in 1944 were still alive and 159 were dead.

A detailed summary of findings is available with the contractor's final report. This report will summarize some of his major observations.

1. Subjects who died of heart attacks classified as other than myocardial infarction had autonomic balance scores in 1944 which indicated relative sympathetic nervous system (SNS) dominance.

2. Subjects who developed high blood pressure showed SNS dominance in 1944, whereas, subjects who were classified as having low blood pressure in 1971 had autonomic balance scores in 1944 which were indicative of relative parasympathetic (PNS) dominance.

3. In addition to the results already cited, subjects who developed bronchitis, skin rash or hapatitis also showed PNS dominance, whereas, subjects who complained of eczema or periods of anxiety in 1971 showed SNS dominance in 1944.

4. Most of the subjects who reported the presence of "psychosomatic" symptoms showed PNS dominance. Since most of the items checked indicated gastro-intestinal or respiratory complaints, the PNS effect seems reasonable. Interestingly, the only two "psychosomatic" items which showed a significant SNS effect were "severe itching" and "swollen ankles". The correlation between itching and SNS dominance is consistent with the previously reported relationship between SNS dominance and eczema.

Significance: The results of this survey indicate that tests of autonomic balance can predict the occurrence of symptoms or of diseases 25 years later. Although the predictions are not sufficient to be directly applicable, they are sufficient to indicate that such tests are worth considering in a longitudinal test battery.

Proposed Course: As soon as the resources become available, pilot studies will be proposed for inclusion in the Baltimore Longitudinal Study Project.

NICHD Annual Report
July 1, 1972 through June 30, 1973
Gerontology Research Center
Clinical Physiology Branch

Research in the Clinical Physiology Branch continues to be geared toward a physiological and biochemical dissection of mechanisms underlying both adaptive and the deteriorative changes of aging - and, when possible, to accomplish this goal in man. An understanding of the complex mechanisms underlying the processes of "normal aging" is an essential preliminary step to an understanding of the still more complex interrelations of aging and disease. As these complexities are clarified, preventive and therapeutic measures can logically be anticipated to follow, with the ultimate goal of achieving the physically and mentally vigorous old age that is now reached by only a small fraction of the population.

The invaluable resource for our study of human aging continues to be the Longitudinal Aging Study. This year has been one of continued development and refinement of unique techniques of longitudinal data analysis. We previously described a new statistical technique which enables the gerontologist to fix (1) the required duration of a longitudinal study, (2) the required frequency of testing for data acquisitions, and (3) the required number of subjects needed in order to define the rates of aging changes in a variety of critical functions with specified accuracy. We can now report the degree of success achieved using the experimental strategies of the current Longitudinal Study. Several examples can illustrate this technique:

(a) To quantitate age changes in the lung, the forced expiratory volume in one second (FEV 1.0) has been measured; the mean rate of aging can be defined in young and middle-aged adults with less than 25% error and in old subjects with less than 10% error.

(b) Blood pressure increases with age show important trends not previously realized. We have recorded blood pressure under truly basal conditions (during basal metabolic rate measurements) and under "casual" conditions, that is, as recorded by a physician during a physical examination. The variance attendant upon the usual casual conditions of blood pressure recording makes it impossible to detect true age changes in systolic pressure with adequate accuracy. Basal systolic pressure on the other hand shows progressively greater rates of increase with advancing age and these age changes can be defined with statistical confidence.

(c) Serum cholesterol and triglyceride levels show strikingly different aging trends. Statistically significant increases occur in cholesterol (errors of less than 25%) in young and middle-aged adults, but level off in late life. Triglyceride levels are constant in young adults and fall significantly (errors again less than 25%) in middle-aged and older men. Reasons underlying the differences in these changes await multivariate analysis of dietary, activity, and other metabolic variables in the coming year.

The versatility of the new analytical technique is best seen when it is used predictively, i.e. for future planning. Again an example can illustrate the principle. In order to define the rate of increase in basal systolic blood pressure in middle-aged adults with great accuracy (defined here as an

error of less than 25%) several experimental strategies could be followed: (1) if an annual examination is performed, 23 years will be required to meet this accuracy goal; (2) if examinations are performed every 18 months, 27 years will be required; (3) if biennial examinations are made, 30 years will be required. Thus for this particular variable, planners of longitudinal studies must think either in terms of a very long-term commitment or in terms of much more frequent examinations than are usually performed. Furthermore we can show that most of the physiological variables of interest to gerontologists will require from 15 to 30 years of study in order to achieve reasonably accurate estimates of rates of aging across the adult age span. This statistical approach in combination with the basic data provided by the Baltimore Longitudinal Study is an achievement which clearly has far-reaching implications for the future.

The difficult problem of separating age changes from disease changes has not been dealt with adequately, either theoretically or practically, by other ongoing studies of human aging. We have devoted much effort the past year to the development of techniques to permit this separation. An automated chart review was the initial step which converted detailed data from medical histories, physical examinations, and laboratory data to an efficiently arranged computerized summary. Multiple-choice entries on histories have been found frequently to be misleading unless the "free field" comments of either the subject or the physician are also considered. These comments are also included in the computerized summary. With these data now available on over 4000 subject-visits, detailed criteria have been developed for the classification and identification of those disease states which could alter physiological functions independent of age. Detailed classifications should provide the standards which other longitudinal studies of human aging will be able to use.

New studies have been introduced into the Longitudinal Study this year. Cutaneous malignancies are a serious problem especially in older subjects. Studies of chronic actinic damage as a precursor of these malignancies have assumed that the damaging radiation spectrum is 290-320 nm and that longer wavelength ultraviolet radiation (320-400) causes "tanning" and may even protect against sun damage to skin. A comprehensive study on the effect of age on sensitivity to UV light of different wavelengths and on the time course of these radiation effects is therefore underway. Results to date show that the longer UV wavelengths actually have an augmentative effect, not a protective effect; thus present preparations to prevent sun damage should be modified so that they protect against a fuller spectrum of UV light. Furthermore, the studies show that individual responses to radiation can be determined in a much shorter period of time than had previously been thought possible; this may have implications for future screening techniques.

New genetic markers have been introduced into the longitudinal study in order to assess further the role of genetics in the aging process. We are now measuring haptoglobin, ceruloplasmin, and transferrin in plasma and are obtaining dermatoglyphic analysis on all subjects.

An interesting analysis have been made of various estimates in use for the determination of body fatness. We have obtained not only heights and

weights, but also the detailed Behnke Index (which includes the abdominal girth), and skin-fold thicknesses. By taking advantage of the longitudinal nature of the data, we have available many instances of changes in body weight, both gains and losses, which in our individual subjects are due almost entirely to spontaneous changes in caloric intake on their part. Thus changes in body weight largely reflect changes in obesity rather than changes in lean body mass. Changes in the individual indices of body fatness were correlated with changes in body weight in order to test which of the measurements was the best index of changes in obesity tissue. The rather surprising result was that changes in skin fold thickness correlated rather poorly with changes in body weight with an r of only 0.4; the complex Behnke Index correlated better with r values of 0.64 - 0.78 in subjects in different age groups, while the best indicator was the simple measurement of abdominal girth which correlated between 0.64 and 0.90 in subjects of different ages. The inadequacy of height-weight tables of judging obesity has been commented upon repeatedly and generally skin-fold thickness measurements have been recommended as a simple addition to the measurement of height and weight in epidemiologic studies in order to improve the accuracy of the esimate of obesity. It would appear that abdominal girth is a distinctly better measurement of fatness than is skinfold thickness.

The nature of the age decrements in myocardial performance has been explored by studies using the isolated trabeculae carneae of young, middle-aged, and old rats. In these perfused preparations, single and paired stimulation has been given under conditions of exposure to graded levels of norpinephrine and to hypoxia. Biophysical muscle responses have been quantitated. The oldest animals show a reduction in developed tension and in the maximal rate of tension development in response to catecholamines. No age differences occurred in the paired-stimulation and hypoxia studies. The successful development of this experimental procedure and the demonstration of age differences in response to catecholamines in these heart muscle preparations will lead to further pharmacologic studies on the role of the sympathetic nervous system in determining age differences in responses of the heart to stress.

Studies have also been carried out on the intact dog heart to test the hypothesis that there is a damaged but salvageable area of heart muscle after coronary ligation. The experimental variables in this complex, carefully controlled preparation are (1) myocardial damage as quantitated by ST segment surface mapping, (2) intramyocardial oxygenation using a polarographic method, (3) coronary artery perfusion pressure. Results show significant correlation between the ST segment map and myocardial oxygen tension on a minute-to-minute basis in each dog. Furthermore increasing the coronary perfusion pressure significantly improves oxygenation and decreases the area of ST segment abnormality. There is thus now experimental evidence (1) to support the validity of using ST segment changes as quantitative indices of the ischemic zone and (2) to support the rationale of elevating coronary perfusion pressure to reduce infarct size.

Study of the chemistry of human renin, a kidney enzyme involved in the pathogenesis of hypertension, has progressed during the past year through

the use of a new labeled polymeric substrate assay previously developed in the Endocrinology Section. We have discovered that the enzyme is inhibited by a variety of proteins and peptides. Studies of the mechanism of this inhibition with a model protein, hemoglobin, with hemoglobin chains, and with chemical and enzymatic cleavage fragments of hemoglobin have shown that large peptides are the most potent inhibitors, but that peptide fragments are also effective. The leucine-leucine bond, which is necessary for renin action on its substrates, is not necessary for inhibition by hemoglobin fragments. A number of small synthetic peptides have also been discovered to be potent inhibitors of renin. Structural requirements for inhibition have been studied. Renin preparations, free of peptidase activity, have been shown to contain protease activity against hemoglobin. A new concept of renin emerges in which renin shares many of the characteristics of only relatively specific acid proteases of wide biologic distribution. The techniques and concepts recently developed in our laboratory should permit the development of renin inhibitors of potential clinical usefulness. They will also allow the development of affinity chromatography procedures which could result in the isolation of the enzyme in pure form.

The possible involvement of the adenyl cyclase ("second messenger") system in senescence is being explored. This membrane-bound enzyme is activated as the initial step in the action of many hormones. As a result, cyclic adenosine monophosphate (cyclic AMP) is formed; a variety of metabolic processes are then initiated by this compound. Although hints of involvement of this system in certain age-related alterations of hormone action can be extracted from the literature, no actual studies of the enzyme in senescence have been reported to date. Our preliminary data now indicate that, in rat liver, senescence is associated with a marked increase of activation of adenyl cyclase by epinephrine. Although non-specific activation with fluoride is also somewhat enhanced, activation by another hormone, glucagon, is unaffected. These observations indicate the existence of hormone-specific effects of age on membrane structure and function. Moreover, they suggest that altered cyclase responses may provide a molecular basis for the altered sensitivity to hormones which has been seen with aging. This line of investigation is being extended to include a variety of hormone-activated cyclase responses in a number of tissues.

The previously developed model for insulin and glucose metabolism in man has been used to compute changes in glucose content of two of the glucose compartments when changes in glucose concentration are induced in the third compartment (the blood volume). This computation is critical especially when large changes in blood glucose concentration occur rapidly, as in the hyperglycemic clamp experiments. Since one of the experimental variables computed in these studies is glucose utilization by the body, the glucose which has been infused but which has gone only into the extracellular glucose spaces must be subtracted from the total glucose infusion rate. This computation could not be made with any degree of accuracy until a successful glucose model was developed.

The complex biphasic response of insulin secretion in response to hyperglycemia has now also been treated mathematically by the development of

a model of the pancreas. The pancreas model behaves "correctly" in terms of its release of insulin in response to hyperglycemia and it can also reproduce the different insulin responses in young and old subjects. Factors influencing insulin secretion other than glucose concentration and age can also be accounted for by the model. The next goal is the incorporation of the pancreas model into the previously developed glucose and insulin models of the body to close the loop of these interrelated variables.

The glucose-clamp technique (both the hyperglycemic clamp to study beta cell sensitivity and the euglycemic clamp during insulin infusion to study sensitivity to insulin) has been used in a prospective study of the mechanisms underlying the glucose intolerance of uremia. The differentiation of uremic glucose intolerance from diabetic renal disease with uremia is important in terms of decisions concerning acceptance of patients into dialysis and transplantation programs; diabetics with uremia have such poor prognosis that they have been given very low priority in these programs. Eight uremic patients have been studied. Seven of them showed resistance to insulin, a mechanism for glucose intolerance which is clearly different from true diabetes mellitus. Four subjects have been studied both before and after chronic hemodialysis. All four showed marked improvement in glucose tolerance as their uremia was ameliorated. The improvement in tolerance could be accounted for by an increase in sensitivity to insulin in three patients and by an increased secretion of insulin in the fourth. Therefore, while both factors may play a role in uremic glucose intolerance, insensitivity to insulin appears to be the more important. Glucose intolerance in uremics thus should not per se make subjects ineligible for definitive therapeutic programs since it frequently is not indicative of true diabetes mellitus.

Studies carried out in collaboration with Baltimore City Hospitals and Johns Hopkins University scientists have increased our understanding of myeloma kidney. Thirty six patients have now been studied prospectively. Changes in urine concentrating and acidifying ability preceded GFR changes. PAH clearance decreased out of proportion to decreases in GFR. Only patients with free Bence-Jones protein demonstrated impaired tubular and glomerular function. Renal biopsy showed that changes in renal function correlated closely with tubular atrophy and degeneration, but not with tubular casts. It appears than that Bence-Jones protein exerts a direct toxic tubular effect and that glomerular dysfunction occurs only after severe tubular damage has occurred.

An interesting patient has evolved from this study, the first successful renal transplant in a myeloma patient. A long-term remission of the myeloma was induced by cyclophosphamide therapy but renal failure persisted. After nine months of dialysis, cadaveric transplantation was accomplished now with successful function 14 weeks later. Myeloma is thus no longer necessarily to be considered an absolute contra-indication to transplantation, if favorable responses to chemo-therapy result in what would otherwise be terminal renal failure.

Serial No. HD-AG4(c)
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Age Changes in Human Performance

Previous Serial Number: Same

Principal Investigators: Ronald Fine
Alan Zuckerman

Other Investigators: N. W. Shock
A. H. Norris

Cooperating Units: Baltimore City Hospitals
Dr. A. T. Welford
Department of Psychology
The University of Adelaide
Adelaide, South Australia

Man Years:

Total:	2.40
Professional:	1.40
Other:	1.00

Project Description:

Objectives: This project is designed to study the effects of aging on: (a) pulmonary and cardiovascular responses to exercise, (b) the rate of recovery of physiologic equilibrium after exercise, (c) efficiency of muscular exercise and (d) work output and fatigue in man. Detailed evaluation of pulmonary function provides a basis for identifying a major factor in the limitation of work performance. In addition, neuromuscular and cardiovascular limitations are assessed.

Methods Employed: Measured amounts of physical work are obtained in subjects of varying ages by means of a calibrated arm ergometer and quantitative mechanical analysis of limb movement. A treadmill is used to induce higher levels of work. Measurements of oxygen uptake, CO_2 elimination, pulmonary ventilation volume, heart rate, blood pressure, and electrocardiogram are made before, during and after standardized amounts of exercise. In addition, the volume of the lungs and functional capacities of the pulmonary system are evaluated. Other studies include measurements of speed of nerve conduction, reflex latency and muscle action potentials.

Major Findings: During the year, analysis in relation to this project has primarily focused upon pulmonary function measurements.

Two physicians independently classified study participants with respect to diagnosed diseases and deformities which could modify performance on pulmonary function tests. Subjects falling into this category constituted about 17% of subjects in age groups 25-34, 35-44, 45-54, 55-64 and about 35% of those in age groups 65-74, 75-84 and 85-94. On the average, subjects in the disease group had lower maximum breathing capacity (MBC) and lower vital capacity (VC) than subjects without evidence of disease. The identification of subjects without significant disease thus permits an evaluation of the effects of aging processes per se on pulmonary function without confounding effects due to disease and other non-aging factors. It also permits other variables which might influence the rate of pulmonary aging independent of recognizable disease to be evaluated.

Smoking, for example, has been reported as having a significant effect on both MBC and VC. When comparisons between smokers and non-smokers were limited to the non-disease group, differences attributable to smoking were still present even in this clinically "pure" group.

Thus, the deteriorative impact of cigarette smoking on pulmonary function is demonstrable in subjects who do not yet manifest evidence of bronchopulmonary disease by history, physical examination, or chest x-ray.

Maximum breathing capacity was found to be significantly related to maximum working power of the arms and shoulders of participants in the longitudinal studies. Since both maximum work and MBC decrease with increasing age, a partial correlation with age held constant was computed (partial $r = 0.37$). The strength of this relationship suggests that a significant portion of the decline with age in MBC is attributable to a loss of muscle and loss of muscular coordination. The remainder of the decline with age is attributable to other changes in lung and chest wall. Consideration should, therefore, be given to muscle and motor status in the interpretation of the MBC test for pulmonary diagnostic purposes.

An automated on-line data collection system has been developed to improve the accuracy of the pulmonary function measurements, eliminate transcription errors, provide immediate feedback to the technician, and facilitate new approaches to data analysis. All of the raw data will be preserved for subsequent reanalysis by new methods should they become of clinical interest in the future. Tests to be processed by the automated system include: Vital Capacity, Forced Vital Capacity, Maximum Ventilatory Volume, and Nitrogen Washout.

Significance to Bio-Medical Research and the Program of the Institute: The decline of the ability of some older people to perform their day-to-day activities and to engage in pursuits which contribute to the economic and social strength of our society represents a national loss. Identification of the physiological, medical and social correlates of high levels of physical strength and psycho-motor performance in middle and old age, as well

as declines in these abilities, should lead to techniques designed to reduce the rate of decline in performance capacities with age.

Proposed Course: Appraisal of participants in the longitudinal studies will be based on measurements of muscle strength in maximum power generating ability during arm exercise. Cardiovascular, ventilatory and metabolic responses to standardized arm ergometer exercise and monitored treadmill exercise will be used to classify participants into fitness categories. Measurements of lung volumes and uniformity of pulmonary ventilation will be made to characterize the respiratory competence of the longitudinal studies participants and to evaluate the benefits of the cessation of smoking in this group.

Honors and Awards: None

Publications: None

Serial No. HD-AG3(c)
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Longitudinal Studies of Human Physiology, Biochemistry and Psychology

Previous Serial Number: Same

Principal Investigators: N. W. Shock, Director
R. Andres, Clinical Director

Other Investigators:
D. Arenberg
B. D. Bricker
M. Butler
B. T. Engle
R. Fine
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Man Years:

Total:	14.15
Professional:	4.65
Other:	9.50

Project Description:

Objectives: This project is designed to: (1) secure replicate measures of physiological, pathological, biochemical and psychological variables on longitudinal study participants at specified intervals; (2) summarize and compare the results of testing in relation to age according to cross-sectional and longitudinal formats; (3) identify factors which may be related to changes of function over time and to age at death; and to (4) determine whether the data obtained support one or another theory as to the mechanisms responsible for age-related functional decrements.

Methods Employed: The Sample: Study participants are all male volunteers recruited by subjects already members of the program. Recruits agree to return to GRC in Baltimore for 2-1/2 days of testing at 12 or 18 month intervals, depending on age, for an indeterminate period of years. At entry into the program, 87% of subjects reported at least some college, 87% were identified with professional, technical or managerial occupations, 90% were presently married, 81% described themselves as financially comfortable, and of the group who returned for the fifth visit, 90% had rated their health as good or excellent on both first and fifth visits.

Testing procedures: Since inception of the study, a major objective of data collection has been to secure uniformity in testing procedures. In the area of psychological testing, standardized tests of personality, intelligence and performance are employed in addition to especially-designed procedures to assess verbal-learning and problem-solving abilities. Systematic methods have guided inquiry into past smoking habits, the dietary history and areas of behavioral experience. Physical examinations and medical histories are performed with check lists to insure completeness, while the variables generated by tests of kidney function, pulmonary function, neuromuscular function, cardiovascular function, and tests of vision, hearing and reaction time follow well-defined and generally accepted procedures.

Data management: In addition to an implied obligation to protect personal privacy and to submit reports of the physical examination to designated personal physicians, primary emphasis is given to the derivation of 'best measures' which are posted for purposes of keypunching and verification. These data are then transferred into the computer file, but before being utilized, are reviewed for completeness and reasonableness. A further review is performed by summarizing test results by age groups and calendar years to be certain of the consistency of statistical parameters over the course of the study.

Major Findings: By April 4, 1973, 960 male subjects had completed one or more visits to GRC, accounting for a total of 4419 participant visits since

the beginning of data collection. As of this date, 657 subjects were classified as being of active status, 1 had requested inactive status, 106 had deceased, 82 had formally withdrawn from the study and 114 no longer responded to inquiry.

In a tabulation designed to compare study subjects born in different decades according to social class, three socioeconomic indicators were employed: years of school completed, highest degree received and usual occupation. The percentage distributions over birth cohorts proved remarkably uniform, considering the fact that males in their twenties and thirties still have an opportunity to complete advanced degrees and to move into technical, professional and managerial occupational positions. Thus, the results help resolve the question of whether our recruiting procedures may have resulted in the inclusion of younger subjects with different socioeconomic characteristics.

A small statistical program library is being developed for use on the on-site Raytheon 706 minicomputer to facilitate analysis of the large volume of data now on file. These programs should significantly reduce computing costs and improve turnaround time for many analyses. The fact that this system is compatible with existing programs at DCRT makes it feasible to transfer intermediate data tapes between the two systems. However, the primary master file will be maintained at DCRT to perform analyses which are too complex for the smaller computer to handle.

The analysis of data from several segments of the Longitudinal Study is dependent upon appropriate clinical classification of the study participants. From the inception of the study, detailed histories and physical examinations have been recorded at the time of each visit using multiple choice questionnaires supplemented by liberal use of free-field comments. During the past year computer programs have been developed to facilitate use of this data which has been previously keypunched, edited, and entered into the Master File of the Longitudinal Study. The computer-generated chart abstracts average two or three pages in length (as compared to two or three volumes for the original hand-written charts) and have been successfully used to complete clinical classification for Cardiovascular, Renal, and Pulmonary diseases for each of nearly 1000 participants in the study.

Only the positive findings on history or physical exam of particular interest to the investigator is included in the computerized summary along with the time course of the finding and any associated free-field comments. The availability of these summaries markedly reduce the time necessary to complete the classifications and also improved their accuracy. The classifications themselves were assigned by the individual investigator who read the summaries and not by a computerized diagnosis algorythm. A computerized diagnostic program could not have dealt with the complexity of the free-field comments, which significantly improved the quality of the data gathered by multiple choice questionnaires. This detailed review of the clinical data, gathered as part of the Longitudinal Study, has led to plans for improving the quality of these data during the next data collection cycle which will begin in July, 1973. Preliminary work for developing on-line automated

patient interviewing systems has also been undertaken as an outgrowth of the Automated Chart Review Project.

Although creatinine clearance has been shown to decrease with age, it has been questioned whether this decrease represents time aging per se or the adverse effect of common diseases which become increasingly prevalent as age advances. These diseases include nephrolithiasis, prostatism, hypertension, urinary tract infections, diabetes, and arteriosclerotic cardiovascular disease. We have, therefore, categorized each visit on each subject in the Baltimore Longitudinal Study with respect to those clinical disorders which are known to influence creatinine clearance. A group of 571 men remained who had none of these disorders. Despite this intensive "purification" of the population, a striking effect of age was present. There was a progressive decline in clearance, decade by decade, from a level of 139 ml/min/1.73 m^2 surface area (± 22 SD) at age 30 to 96 ± 20 at age 80. Analysis of symptoms of prostatism and prostatic size showed no effect of these variables on creatinine clearance in this population. These data, from a large, intensively studies group, define normative age-corrected standards for creatinine clearance in man.

The Longitudinal Study has provided some unique information on blood pressure and aging. Blood pressure is measured in the usual fashion as part of the physician's physical examination, and in addition, basal blood pressures are obtained during standardized BMR conditions. These measurements are referred to as "casual" and "basal" blood pressures. Analyses of age differences between casual and basal pressure and in pulse pressures under these conditions have now been made. The clinical classification scheme used for creatinine clearance was applied to the blood pressure data to minimize the impact of age-related diseases on intrinsic aging processes. On 581 subjects who were defined as normal, a clear-cut "stress effect" of the first examination on blood pressure was shown on a longitudinal visit-to-visit analysis of pressure changes; the first visit pressures were clearly above the blood pressure slopes established by measurements on subsequent visits. True aging trends in blood pressure, therefore, must be computed omitting the first data point.

Age-associated increase in systolic, diastolic, and pulse pressure was found as expected. Of importance was the finding that casual pressures exceeded basal values by about 8 mm systolic and 5 mm diastolic from ages 30 to 60 but this excess of pressure under casual conditions fell to about 6 and 2 mm respectively in the 70 year old age group.

These data are of value in interpreting blood pressure data obtained under differing experimental conditions. Thus, the critical levels reported by the V. A. Cooperative Study Group Trial and measured under resting conditions may be at least 8/5 mm lower than the casual values obtained in a physician's office.

The planning of longitudinal studies involves decisions concerning the total duration of the study and the frequency of testing. Such decisions have usually depended upon educated guesses rather than upon rational statistical

principles. An experimental strategy which will result in the accurate definition of the rate of change (slope) in a function depends upon (1) the magnitude of the slope, and (2) the variance in the data points. Thus, a function which changes strikingly with age and in which the data points comprising the individual slopes show little variance, can be defined with reasonable accuracy in a relatively short period of time with relatively infrequent observations. The obverse is equally true. Using statistical methods recently developed at NICHD, and using data from the Baltimore Longitudinal Study, we can now provide estimates of appropriate strategies for a variety of functions which are of interest to physiological and clinical gerontologists. The implications for the planning of ongoing and future longitudinal studies as well as for possible therapeutic intervention studies are clear: on the usual annual or biennial testing schedule, most functions will require about 15 years of testing in order to define aging rates with acceptable accuracy.

Significance to Bio-Medical Research and the Program of the Institute: A major goal of the Longitudinal Program is a deeper understanding of age-related changes in the different organ systems, and their interrelationships. In this regard, the identification of coronary artery disease serves to characterize an age-related disease in the cardiovascular system. Numerous studies elsewhere have demonstrated that heart disease occurs with increased frequency in those who are hypertensive, inactive, obese or diabetic and in those who smoke cigarettes or have elevated blood lipid levels. The potential contribution of our Longitudinal Study to this important public health problem lies in the intensity of study of a number of these variables in this study.

Collection of this body of data will place us in a position to test theories of aging with the human population of the longitudinal studies. Results of comparisonsbetween old and young individuals are presently available, but these do not permit us to evaluate theories of aging adequately. Our ability to do so will depend in part upon a comparison of the integrated medical , physiological, psychological and biochemical profiles of the individual participant with various subsequent outcomes which we may be in a position to monitor in the same individual. Outcomes such as length of life, occurrence of disease, rate of change in a functional parameter, among other things, will be sought. Some theories assume that a few cells are lost during each unit of time so that in an individual or in an individual organ system changes of a gradual nature would be found. These theories are consistent with the summation of a series of distributed minor accidents (such as radiation hits) if only a few cells are involved each time. Other theories would relate large changes in function with the occurrence of a disease episode, or major accident resulting in a substantial loss of cells. These theories are consistent with abrupt changes in functional levels which would be maintained until another major accident results in a new level. In addition, these two widely different types of theories could be found in combination with sudden as well as slow changes operating simultaneously.

Proposed Course: Data collection will be continued as in the past, except as new equipment may provide greater capacity for accurate determinations and for automating procedures. The fact that automated procedures for

testing auditory reaction time and for measuring numerous parameters of pulmonary function have now been implemented will greatly aid the achievement of the above objectives.

Honors and Awards: Dr. Shock received the American Pioneers in Aging Medal from the Institute of Gerontology at the University of Michigan--Wayne State University, 1972.

Dr. Shock received the First Annual Award for Outstanding Contributions to Bio-Medical Sciences and Aging from the Ethel Percy Andrus Gerontology Center at the University of Southern California, 1973.

Publications: Shock, N. W.: Gerontology - past, present, and future. In: D.F. Chebotarev, V. V. Frolkis, and A. Ya. Mints (Editors), Proceedings of the 9th International Congress of Gerontology, Vol. 1, Plenary and Section Sessions. The Congress, Kiev, U.S.S.R., July, 1972, pp. 13-21.

Andres, R., and J. Tobin: Aging, carbohydrate metabolism and diabetes. In: D. F. Chebotarev, V. V. Frolkis, and A. Ya. Mints (Editors), Proceedings of the 9th International Congress of Gerontology, Vol. 1, Plenary and Section Sessions. The Congress, Kiev, U.S.S.R., July 1972, pp. 276-280.

Tobin, J. D., R. S. Sherwin, J. E. Liljenquist, R. A. Insel, and R. Andres: Insulin sensitivity and kinetics in man. (Abstract 435) in: D.F. Chebotarev, V. V. Frolkis, and A. Ya. Mints (Editors), Proceedings of the 9th International Congress of Gerontology, Vol. 3, Abstracts. The Congress, Kiev, U.S.S.R., July 1972, p. 155.

Shock, N. W.: Energy metabolism, caloric intake and physical activity in the aging. In: L. A. Carlson (Editor), Nutrition in Old Age (X Symposia of the Swedish Nutrition Fdn.), Almqvist & Wiksell, Uppsala, 1972, pp. 12-23.

Shock, N. W.: Gerontology and geriatrics. In: Encyclopaedia Britannica. Encyclopaedia Britannica, Inc., Chicago, 1973, pp. 363-365.

Shaw, D. J., D. A. Rothbaum, C. S. Angell, and N. W. Shock: The effects of age and blood pressure upon the systolic time intervals in males aged 20-89 years. J. Gerontology, 28: (2), 133-139, April 1973.

Serial No. HD-AG5(c)
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Age relationships of body composition, nutrition and physical activity

Previous Serial Number: Same

Principal Investigators: Reubin Andres
N. W. Shock

Other Investigators: Marlene Butler
Arthur H. Norris

Cooperating Units: Baltimore City Hospitals

Dr. Paul Gyorgy
Philadelphia General Hospital
Department of Public Health
Philadelphia, Pennsylvania

Dr. Myron Brin
Assistant Director for Clinical Nutrition
Hoffman-LaRoche, Inc.
Nutley, New Jersey

Professor George Vose
Research Institute
Texas Women's University
Denton, Texas

Dr. Harald Schraer
Department of Biophysics
Pennsylvania State University
University Park, Pennsylvania

Man Years:

Total:	2.15
Professional:	.15
Other:	2.00

Project Description:

Objectives: This project is designed to describe differences in body composition, nutrition, and physical activity between males of diverse age, examine these differences in relation to age, and to determine whether such differences may influence physiological and biochemical characteristics of individual subjects.

Methods Employed: Height, weight, and body circumferences of longitudinal study participants are obtained by standard anthropometric methods. Roentgenographic and anthropometric estimates of skeletal mass are combined with height, weight, and body circumferences to provide an estimate of body fat. Other estimates of fat include skinfold thickness measurements and fat thickness measurements from X-rays. Estimates of lean body mass include: (1) basal metabolic rate determinations made with standard open circuit methods, (2) twenty-four hour urinary excretion of creatinine, and (3) total body water and extracellular water determinations made from the distribution curves of injected antipyrine and sodium thiocyanate. Nutrient intakes and activity calories are estimated from interviews and self-administered questionnaires. All such measurements are repeated according to schedule in the course of each subjects participation in the longitudinal program.

In a collaborative study with nutritionists at Philadelphia General Hospital and at Hoffman-LaRoche, Inc., the status of three vitamins of the B complex are assessed. Excretion levels of thiamine (B_1) and riboflavin (B_2) are obtained from a 24 hour urine collection. Transketolase activity and glutathione reductase activity in red blood cells are measured and serve as additional indicators for adequacy of thiamine and riboflavin respectively.

Tests for pyridoxine (B_6) status include determinations of pyridoxal phosphate in plasma and glutamic oxalic transaminase in plasma and in erythrocytes. Amino acid load tests using tryptophan and methionine are also given. One-half of the subjects receive tryptophan in an amount equal to 25 mg per kg body weight. A subsequent urine collection is analyzed for xanthurenic acid. The rest of the subjects receive methionine, 50 mg per kg body weight. The urine collected after the methionine load test will be analyzed for cystathionine and other methionine metabolites.

Major Findings: The collection of blood and urine samples for purposes of the B vitamin study has been completed for 620 men, 91% of the active longitudinal studies population. The tentative conclusions made last year have been strengthened and no further data collection is planned at this time. Manuscripts are in preparation.

Interrelationships of measures of fat and lean body mass have been computed in the longitudinal studies population. Indices of fatness were skinfold thicknesses, abdominal circumferences and an estimate of fat based on the Behnke Anthropometric Index. Indices of lean mass were basal metabolic rate, 24 hour creatinine excretion, intracellular water, biceps circumference and the Behnke Index. Stature, arm strength and caloric expenditure

attributable to physical activity were also considered (1) in a correlation analysis and (2) as predictors of change in body weight.

To avoid confounding of the relationship between paired variables because of a common relationship of the variables to age, partial correlation coefficients were computed with age held constant. Correlations among variables which estimated fat were significant (partial r = 0.46 - 0.61). Similarly, variables which estimated lean body mass were significant (partial r = 0.34 - 0.50). As expected, total body weight was strongly related to both fat and lean mass estimators (partial r = 0.46 - 0.84). In addition, abdominal circumference was strongly related to both fat and lean mass estimators (partial r = 0.49 - 0.84). Stature, arm strength and activity calories were not significantly related to fat and lean mass estimators. Stature was related to total body weight (partial r = 0.47).

The group of estimators of fat were interrelated as were the group of estimators of lean body mass. There were no correlations between the estimators of lean mass and estimators of fat. Body weight and abdominal circumferences were related to both groups of estimators.

The relative usefulness of these estimators of body mass for estimation of change in body fat was evaluated. It was assumed that weight changes between visits(12 to 18 month interval) represented gain or loss of fat. This is probably a valid assumption for this relatively sedentary population. Visit by vist changes in weight were compared to changes in estimator variables for corresponding visits. Correlations between Δ weight and Δ estimator were calculated for young (15-44 years), middle aged (45-64 years), and old (65 or more years) subjects. The best estimator of change in weight was abdominal circumference, r = 0.90 in young subjects to r = 0.60 in old subjects. The second best estimator was the Behnke Anthropometric Index, r = 0.78 in young to r = 0.64 old.

Change in skinfold thickness and change in weight were correlated with an r of only 0.37 and there was no difference between young and old subjects in this relationship. The surprising finding that change in abdominal girth reflects change in body weight better than does skinfold thickness suggests that the addition of abdominal girth to height and weight measurements in epidemiological studies may yield a better index of fatness than will skinfold measurements.

Significance to Bio-Medical Research and the Program of the Institute:
Nutritional deficiencies in the aged are known to be common and are generally attributed more to the socio-economic deprivation of this group than to biological or physiological aging effects. The volunteers in the Longitudinal Study Group under investigation at the Gerontology Research Center are not a deprived group--it may be characterized as upper-middle class and has a very high educational level. It, therefore, offers a unique opportunity to study nutritional status under very favorable conditions. The nutritional effects of biological aging per se may therefore be separated from what might be called "social aging."

Certain changes in organ systems with advancing age and various entities

of disease are thought to be affected by diet, levels of physical activity and body composition. From the repeated assessment of these factors over time, it may be possible to determine their relative contributions to longevity and the maintenance of health and vigor in later life. Difficulties associated with obtaining estimates of eating habits, activity and body composition in the past make a prospective approach necessary for the collection of reliable information.

Proposed Course: Prediction equations for suggested estimators of fat and lean body mass will be developed. Interrelationships of changes in body composition and changes in physical activity and food intake will be explored.

Future plans for the B vitamin study are to complete the laboratory and the statistical analyses on the 620 men. Repeat tests available on 160 subjects afford an opportunity to study the variance of the measurements within individuals.

Mean daily intakes of thiamine, riboflavin, and pyridoxine, as calculated from a seven-day food intake record, as well as amounts of these three vitamins ingested daily from vitamin supplements, will be examined for age differences and these intakes will be correlated with the biochemical estimates of B vitamin status.

Correlations between urine tests and blood tests for the same vitamin will be done for each category of adequacy; low, normal, and high.

Subjects will be classified according to the presence of several diseases which may be related to vitamin deficiency and each group will be examined for differences in vitamin status.

Studies of diet, physical activity, and body composition will continue to be performed according to schedule. Correlations among the several estimates of lean body mass and of obesity will be further analyzed. The power of these estimates in predicting certain deleterious aging variables will provide evidence of the relative value of these measurements.

Honors and Awards: None

Publications: None

Serial No. HD-CP2(1)(c)
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Identification and characterization of cerebrovascular disease in Longitudinal Participants and correlations with other parameters of physical and psychological function.

Previous Serial Number: Same

Principal Investigator: C. Paul Scott

Other Investigators: Arthur H. Norris
Reubin Andres

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	0.00
Professional:	0.00
Other:	0.00

Project Description: This project has been incorporated into the project: Longitudinal Studies of Human Physiology, Biochemistry and Psychology, Serial No. HD-AG3(c). The classification system developed under this project is being continued for later evaluation as data accumulate.

Honors and Awards: None

Publications: None

Serial No. HD-CP4(9)(c)
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pulmonary Function Studies: Spectral analysis of cardio-pulmonary parameters as a means of quantifying cardio-pulmonary function

Previous Serial Number: Same

Principal Investigator: Ronald Fine

Other Investigators: Phillip Thorne
Arthur H. Norris

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	0.00
Professional:	0.00
Other:	0.00

Project Description: This project has been discontinued. The technical and computational methods required could not be solved.

Honors and Awards: None

Publications: None

Serial No. HD-AG1(c)7
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1973

Project Title: Marital, Sexual and Social Factors in Aging

Previous Serial Number: Same

Principal Investigator: C. Martin

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	0.20
Professional:	0.20
Other:	0.00

Project Description:

Objectives: Major goals are to: (1) describe the effects of age and generation on marital adjustment, sexual reactivity and sexual performance, (2) determine whether individual differences in sexual functioning may be related to some aspect of health, physiologic state or psychologic trait, and to (3) discover whether differences in marital, sexual and social background may be associated with specific diseases or longevity.

Methods Employed: Each subject in the course of participating in the longitudinal study, is asked to contribute an interview dealing with his history of marriage and sexual activity. Subjects are further questioned with respect to parental-home experience, military service, occupational and educational history and with respect to indicators of emotional stress. In requesting the interview, the investigator outlines the objectives of the study, provides assurance regarding confidence and emphasizes the voluntary nature of such a contribution. By now 609 subjects have completed interviews, another 16 have partially completed interviews while 14 declined to be interviewed. Resulting data are incorporated into the computer master file.

Participants are also asked to complete the Activities and Attitudes Questionnaire on their first, fifth and ninth visits to GRC. The questionnaire, initially designed to provide scores indicative of personal adjustment, contains many items concerning demographic, attitudinal and

behavioral kinds of information. Coded responses to these questions are regularly updated in the master file.

Major Findings: Utilizing the contingency table computer program, variables derived from the above sources of data were subjected to two types of analysis. In the first, or cross-sectional type, variables were tabulated according to age for evidence of changes with age. Illustrative data from the interview, shown below, indicate how current status of coital potency tends to decline with advancing age. This finding will hardly occasion surprise although the data are unique in that systematic information pertaining to current status of erotic reactivity, coital activity and erectibility were used to derive the set of mutually exclusive and exhaustive categories. It should be noted that such data provide no evidence as to why some men become impotent or partly so with increasing age while other men do not.

Current Status of Coital Potency

Current Status	Age at Interview				
	20-39	40-49	50-59	60-69	70-79
Number of subjects	75	128	151	100	83
	Percentages				
Never or incidental failure	92.0	89.1	80.8	62.0	44.6
Occ/freq failure to obtain	2.7	2.3	4.0	4.0	6.0
Occ/freq failure to maintain		3.1	4.6	8.0	9.6
Occ/freq both types of failure	1.3	3.1	4.6	6.0	7.2
Absent: eroticism or erectility			1.3	11.0	15.7
Indeterminate: no recent coitus	4.0	2.3	4.6	9.0	16.9
Total	100.0	99.9	99.9	100.0	100.0

The second type of analysis explored the statistical relationships between key variables when age was held constant. The example shown below describes how the two variables, age at first coitus and number of coital partners before age 40, are strongly correlated among subjects falling into two age groups. Since this relationship had not previously been noted, these data are again unique. Findings of this kind can contribute to a better understanding of male sexuality, and knowledge of this relationship may well prove useful for interpretation should either of these variables appear as an important correlate in subsequent analyses.

Age First Coitus	Interviewed at Ages 40-59 No. Coital Partners before Age 40				Interviewed at Ages 60-79 No. Coital Partners before Age 40			
	0-1	2-4	5-8	9+	0-1	2-4	5-8	9+
< 18		8	21	40		2	2	22
18-20	4	29	22	37	3	13	12	15
21-24	28	30	13	6	21	13	6	4
25-49	33	6	3	1	61	9		2
Total	65	73	59	84	85	37	20	43

Significance to Bio-Medical Research and the Program of the Institute: The findings reported above and many others involving non-sexual variables, generated by these several approaches to analysis are of descriptive and correlative interest. On the other hand, in the context of an investigation into the processes & vicissitudes of aging, such data are hardly of etiologic interest for they supply no clues to the mechanisms involved. Clearly, other analytic approaches are needed for etiologic objectives.

Proposed Course: The third and next stage of anlysis calls for specifying those variables whose variation over individuals remains to be explained. Here, variables which represent the culmination of some process over time, are related to other variables that are concomittant with or antecedent to endpoint status, for evidence of association. Now that data on prostatic size and symptoms are nearly complete, plans are to relate the four end-point variables: (1) prostatic size, (2) prostatic symptoms, (3) current status of coital potency, and (4) level of recent sexual activity, to one another, to various physiologic variables and to individual differences in marital, sexual and social background characteristics.

Honors and Awards: Dr. Martin was invited to present a paper on November 1-3, 1973 to the Society for Life History Research in Psychopathology.

Publications: None

Serial No. HD-CP 13
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Dermatoglyphics and Clinical Medicine

Previous Serial Numbers: HD-CD1(c), HD-CD45(c), HD-CD48(c), HD-CD17(c), HD-CD49(c), HD-CD51(c), HD-CD52(c), HD-CD53(c), HD-CD43(c), HD-CD44(c)

Principal Investigator: Chris C. Plato

Other Investigators: W. Wertelecki
J. D. Niswander
F. S. Steinberg
J. Cereghino
C. C. Fraumeni, Jr.

Cooperating Units: Department of Pediatrics
Medical University of South Carolina

Department of Human Genetics, NIDR

Program Statistics Branch, NICHD

C & F Research, NINDS

Man Years:

Total:	.50
Professional:	.50
Other:	.00

Project Description:

Objectives: To establish possible statistical associations between various clinical anomalies and dermatoglyphic features.

Methods Employed: Finger and palmprints were collected from patients with cleft palate, congenital heart disease and leukemia, and from their parents and sibs where possible. Prints were also obtained from patients with Down's Syndrome and other types of mental retardation. Prints taken from sibs were to serve as internal controls in addition to panels of white and black Americans used as control subjects. Several methodologies were developed to facilitate detailed comparisons.

Major Findings: Statistical analysis revealed significant relationships between the disease entities of leukemia, Down's Syndrome and deletion of the long arm of the 18th chromosome, and certain dermatoglyphic characteristics.

Significance to Bio-Medical Research and the Program of the Institute: The finding of important statistical associations between various diseases and dermatoglyphic markers may assist in the determination of their etiology.

Proposed Course: To continue this method of investigation taking into consideration other clinical anomalies.

Honors and Awards: Mr. Plato will present a report on Down's Syndrome to the 8th Joint Meeting of the U.S.P.H.S. Professional Associations, in Phoenix, Arizona.

Mr. Plato gave three reports on methods and one on leukemia at the IV International Congress of Human Genetics (Paris).

Publications: Plato, C. C., Cereghino, J., and Steinberg, F.S. Palmar dermatoglyphics of Down's Syndrome. Revisited. Pediatric Research 7:111-118, 1973.

Plato, C. C., and Wertelecki, W. A method for subclassifying the interdigital patterns: A comparative study of palmar configurations. Am. J. Physical Anthropology. 37:97-110, 1972.

Wertelecki, W., Plato, C., Fraumeni, C., and Niswander, J. Dermatoglyphics in leukemia. Pediatric Research. In press.

Serial No. HD-CP 14
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Twin Studies: I. Structure and Color of the Iris
II. X Chromosome Inactivation
III. Dermatoglyphics and Zygocity Determination

Previous Serial Numbers: HD-CD41(c), HD-CD35(c), HD-CD8(c)

Principal Investigator: Chris C. Plato

Other Investigators: J. T. Schwartz
J. D. Mann
J. D. Niswander

Cooperating Units: F & D Research, NEI

Butterworth Hospital
Grand Rapids, Michigan

Department of Human Genetics, NIDR

Man Years:

Total:	.10
Professional:	.10
Other:	.00

Project Description:

Objectives: 1. To determine the degree of variability and heritability of the structure of the human iris. 2. To test the Lyon Hypothesis regarding X chromosome inactivation. 3. To develop, through dermatoglyphics, further criteria for twin zygocity diagnosis.

Methods Employed: Thorough eye examinations, color slides of the irises and finger and palmprints were obtained from over 600 pairs of twins. Zygocity was established by elaborate blood group typing, perinatal information, and physical similarities. Fourteen pairs diagnosed as monozygotic but being discordant for the Xg^a sex linked blood group were involved with their parents and sibs, in the X chromosome inactivation study.

Major Findings: Through our twin studies we demonstrated for the first time that mother-fetus incompatibility, due to the Xg^a blood group, may

result in fetal loss. Because of the sex-linked inheritance of the Xg^a, the fetal loss is confined only to the female fetus.

Significance to Bio-Medical Research and the Program of the Institute: 1. To establish possible associations between iris structure and eye disorders; 2. to provide supporting or rebutting evidence for the Lyon Hypothesis for X chromosome inactivation; 3. to offer additional means for determining twin zygocity.

Proposed Course of Project: To evaluate the data already collected and to perform the appropriate statistical tests.

Honors and Awards: None

Publications: None

Serial No. HD-CP 15
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Dermatoglyphics of Primitive and Developing Populations

Previous Serial Numbers: HD-CD10(c), HD-CD11(c), HD-CD36(c)
HD-CD46(c), HD-CD50(c)

Principal Investigator: Chris C. Plato

Other Investigators: D. Carleton Gajdusek
Ralph Garruto
Robert McLennan
Paul Brown

Cooperating Units: Special Chronic Disease Studies
Department of Anthropology
Pennsylvania State University

C & F Research, NINDS

International Agency for Research on Cancer
WHO, Lyon, France

Man Years:

Total:	.20
Professional:	.20
Other:	.00

Project Description:

Objectives: This project represents part of a multidisciplinary effort in conjunction with the World Health Organization to study various genetic, clinical and anthropological markers among the developing peoples of the South Pacific and South America.

Methods Employed: Dermatoglyphics were collected by the Faurot inkless method.

Major Findings: Dermatoglyphic characteristics are for the most part governed by multiple genes and complex types of inheritance. Consequently, they are not readily vulnerable to the influences of selection or genetic drift due to isolation or migration. Thus, the incidence of dermatoglyphic characteristics found in different populations may reveal the nature of the

relationships existing between populations in the remote as well as the more recent past.

In Cyprus, similarities found with respect to dermatoglyphics and to thalassemia between peoples in the highlands and the lowlands are indicative of an ancient relationship, while wide differences in blood groups suggest recent changes resulting from isolation and genetic drift among highland peoples, and from migration and admixture among the lowland people.

In New Guinea, the Fore (a people affected with Kuru) had different dermatoglyphics and also blood groups than their neighbors, the Anga. The findings indicate ancient and persistent genetic differences due to mechanisms affecting isolation. Among the Quechuas of Peru, dermatoglyphic differences were evident while serological factors were quite alike. In contrast to the Cyprus situation, this latter finding is suggestive of ancient genetic differences which have largely disappeared because of more recent admixture.

Significance to Biomedical Research and the Program of the Institute: The thorough evaluation of genetic and clinical markers of the few remaining primitive populations will allow scientists at a later time to ascertain the effects of "western" culture, infections and environmental pollution upon populations, thus far relatively unexposed to these kinds of influence.

Honors and Awards: Mr. Plato delivered reports at the annual meetings of the American Society of Human Genetics and the American Association of Physical Anthropology.

Mr. Plato also presented papers at the IV International Congress of Human Genetics in Paris.

Publications: Plato, C. C., Brown, P. and Gajdusek, D.C.: Dermatoglyphics of the Micronesians from the Outer Islands of Yap. Zeitscrift fur Morph und Anthrop. (Stutgart) 64:29 44, 1972.

Plato, C. C. and Gajdusek, D.C.: Genetic studies in relation to Kuru: V. Dermatoglyphics of the Fore and Anga populations of the Eastern Highlands of New Guinea. American J. Hum. Genet. 24(supplement): S86-S94, 1972.

Plato, C. C. and Gajdusek, D.C. Dermatoglyphics of the Natives of Western New Guinea. Human Biology in Oceania: 1(4):255-258, 1972.

Serial No. HD-CP 16
1. Clinical Physiology Branch
2. Human Performance Section
3. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Genetic Studies on Longitudinal Program Subjects

Previous Serial Number: New Project

Principal Investigator: Chris C. Plato

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	.20
Professional:	.20
Other:	.00

Project Description:

Objectives: To generate dermatoglyphic, haptoglobin, transferrin and ceruloplasmin data on Longitudinal Study subjects. These genetic markers will be examined with respect to differences in aging characteristics of the studied population.

Methods Employed: Serum protein determinations are carried out by the use of vertical starch gel electrophoresis. Finger and palm prints are collected by the Faurot inkless method.

Major Findings: Data collection was recently initiated. It will require 18 months in order to characterize most of the active participants in the study.

Significance to Bio-Medical Research and the Program of the Institute: Will provide further genetic identification of longitudinal subjects. Repeated evaluations of serum of the same individuals may indicate age changes in the total haptoglobin or haptoglobin-subtype levels. The dermatoglyphics of older subjects, compared to those of our seven-year-old controls may offer evidence of selective advantage for certain dermatoglyphic characters.

Proposed Course: To continue data collection and analysis.

Honors and Awards: None

Publications: None

Serial No. HD-CPl(c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: A programmed stress test for the electrocardiographic diagnosis of coronary artery disease

Previous Serial Number: Same

Principal Investigators: Charles S. Angell
Edward G. Lakatta

Other Investigators: Nathan W. Shock
Reubin Andres

Cooperating Unit: Baltimore City Hospitals

Man Years:

Total:	.20
Professional:	.20
Others:	.00

Project Description:

This test has been systematically administered to participants in the Longitudinal Study. Results are incorporated into the report on the Longitudinal Study.

Serial No. HD-CP6(c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Non-invasive evaluation of ventricular function in man by measurement of systolic time intervals

Previous Serial Number: Same

Principal Investigators: Donald A. Rothbaum - resigned July 1, 1972
Charles S. Angell

Other Investigator: Nathan W. Shock

Cooperating Unit: Baltimore City Hospitals

Man Years: None

Project Description: This project has been completed.

Honors and Awards: None

Publication:

Shaw, D. J., Rothbaum, D. A., Angell, C. S., and Shock, N.W.: Effects of age and blood pressure upon the systolic time intervals in males aged 20-89 years. J. Geront., 28: (2), 133-139, 1973.

Serial No. HD-CP 9
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of coronary perfusion pressure on myocardial oxygen tension and epicardial ST segment changes in acute infarction in dogs.

Previous Serial Number: Same

Principal Investigators: Charles S. Angell
Edward G. Lakatta

Other Investigators: Myron Weisfeldt
Nathan W. Shock

Cooperating Unit: None

Man Years:

Total:	1.0
Professional:	.6
Others:	.4

Project Description:

Objectives: The concept of a compromised yet potentially viable zone of myocardium in an acute infarct forms the basis of current attempts at myocardial salvage by the use of coronary artery bypass grafts, intraaortic counterpulsation, and various pharmacologic interventions. Implicit in these therapeutic maneuvers is an attempt to limit infarct size by restoring to normal the altered balance between myocardial oxygenation and myocardial oxygen demand that is thought to prevail locally in ischemic areas.

The purpose of this study is to evaluate the effects of coronary perfusion pressure on myocardial tissue oxygen tension and the epicardial ST segment map of ischemic injury during the first 15 minutes after coronary artery ligation under conditions of constant aortic pressure and

heart rate. In so doing, the hypothesis that changes in ST segment levels in an ischemic zone reflect myocardial oxygenation on a minute to minute basis is tested as is the hypothesis that elevation of coronary perfusion pressure promotes reduction of infarct size.

Methods: An open chest, intact canine heart preparation is used. Intramyocardial oxygenation is measured using a polarographic technique. The magnitude of ischemic injury is assessed by ST segment mapping. Appropriate catheters are placed for arterial pressure measurement, venous infusion, and arterial withdrawal. The left coronary artery is perfused from a reservoir of arterial blood the height of which determines coronary perfusion pressure.

Experimental observations are recorded under baseline conditions, after coronary artery ligation, after elevation of coronary perfusion pressure and after reduction of coronary perfusion pressure. Appropriate control experiments are performed to evaluate the *in vivo* stability of the oxygen electrode system and to study changes in experimental variables that occur in the absence of changes in coronary perfusion pressure.

Major Findings: The project is 90% completed. Significant correlation ($p<.04$) between intramyocardial oxygen tension and the ST segment map was obtained in every experiment indicating that changes in ST segment levels in an ischemic zone immediately after coronary artery ligation do indeed reflect myocardial oxygenation on a minute to minute basis. The hypothesis that elevation of coronary perfusion pressure promotes reduction in infarct size as assessed by ST segment mapping has also been confirmed as the following table indicates.

	Control CPP 100	$\bar{p}$ Ligation CPP 100	CPP 145	CPP 70
$\overline{p^{O}2}$	15.76 ± 0.80	3.91 ± 0.93 ($p<.001$)	10.42 ± 1.83 ($p<.001$)	4.00 ± 0.92 ($p<.001$)
$\overline{ST}$	1.68 ± 0.3	5.08 ± 1.0 ($p<.02$)	2.37 ± 0.99 ($p<.002$)	4.52 ± 1.13 ($p<.004$)

Significance to Biomedical Research and the Program of the Institute: The importance of infarct size in the etiology of cardiogenic shock as a sequel to acute myocardial infarction has been well-documented, and it is recognized that a possible therapeutic approach to this problem is an attempt to limit infarct size by salvaging ischemic but potentially

viable myocardium. The significant correlation that we have demonstrated between myocardial oxygenation and the ST segment map supports the validity of previous studies using the ST segment map as an index of infarct size. In addition, it appears that the ST segment levels reflect myocardial oxygenation and that coronary perfusion pressure changes produce changes in myocardial oxygenation that influence the viability of ischemic myocardium and ultimately infarct size.

Proposed Course of Project: This project will be completed by June 30, 1973.

Honors and Awards:

Dr. Angell has been invited to present the results of this project to the Division of Cardiology, Johns Hopkins Hospital, Baltimore, at a Myocardial Infarction Research Unit Conference.

Publications: None.

Serial No. HD-CP 10
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Age differences in cardiovascular performance and baroreceptor control mechanisms in intact unanesthetized rats.

Previous Serial Number: Same

Principal Investigators: Donald A. Rothbaum
Charles S. Angell

Other Investigator: Nathan W. Shock

Cooperating Unit: Baltimore City Hospitals

Man Years: None.

Project Description: This project has been completed.

Honors and Awards: None

Publications:

Rothbaum, D. A., Shaw, D. J., Angell, C. S., and Shock, N.W.: Cardiac performance in the unanesthetized senescent male rat. Accepted for publication in Journal of Gerontology.

Serial No. HD-CP 17
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Cardiovascular Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effect of age on the response to inotropic interventions and hypoxia of rat trabeculae carneae

Previous Serial Number: None

Principal Investigators: Edward G. Lakatta
Charles S. Angell

Other Investigators: Nathan W. Shock
Myron Wesifeldt

Cooperating Units: Baltimore City Hospitals and Johns Hopkins Hospital, Division of Cardiology

Man Years:

Total:	.6
Professional:	.6
Others:	.0

Project Description:

Objectives: Recent observations in man and in intact animals have suggested that there are important relationships of catecholamine effect and age. The objectives of this project are (1) to determine if there is an age related difference in the intrinsic response of isolated heart muscle to the inotropic effects of catecholamines, in terms of dose-response relationships, maximal inotropic response, and minimal dosage necessary to produce an inotropic response, (2) to measure the effects of paired stimulation, a non-catechol intervention; these results will be used to determine whether a resulting age difference in the response to catechols might be attributed to the known lower intrinsic level of contractility in the senile rat heart muscle, or whether there is indeed a specific alteration in the response to catecholamines, (3) to determine the rate of decline in performance of cardiac muscle of young and old rat heart muscle following exposure to severe hypoxia and the rate and extent of recovery following reoxygenation.

Methods: Trabeculae carneae were removed from the left ventricles of rat hearts of age 6-, 12-, and 24-27-month-old rats under ether anesthesia. The average cross sectional area of these muscle strips was less than 1 mm^2. The muscles were suspended between two clips and placed in a chamber which was perfused with Krebs-Ringer Bicarbonate solution; this solution was modified by reducing the calcium and magnesium by one half, and by adding dextrose in concentration of 300 mg%. This perfusate was bubbled with 5% CO_2 and 95% O_2 both in the reservoir and directly in the chamber. The temperature was kept constant at 28 ± .5° by a Hooke water circulator. After ninety minutes of equilibration at one gram of resting tension, the length which produced the greatest developed tension was determined (Lmax); after another 60 minutes a baseline value was recorded for the following parameters: Resting tension, developed tension, maximal rate of rise of tension, time to peak tension, and time for peak developed tension to fall 1/2 of its value. The muscle was stimulated at a rate of 24 per minute.

After baseline measurements, the following interventions were made: Paired stimulation was produced by introducing a second stimulus at varying intervals after the initial stimulus over the range of 400 to 120 msec delay. Norepinephrine was infused in five different successive doses (range 1×10^{-8} to 8.5×10^{-5}) allowing 8 minutes equilibration at each dose.

Acute hypoxia was created by withdrawing the oxygenated perfusate from the chamber and replacing it with perfusate which had been bubbled with 95% N_2 - 5% CO_2; the chamber was then also bubbled with this gas mixture. With this method, the PO_2 fell to 25 mm by 10 minutes; the muscles were kept hypoxic for an additional 10 minutes. For reoxygenation, the above sequence was reversed, the PO_2 reaching baseline (PO_2 690 mm Hg) by the end of the first minute.

Following the experiment, cross-section area was determined. The muscle was assumed to be a cylinder and by multiplying the weight by the density (1.063), then dividing the volume by the length cross section area in mm^2 was estimated. The results were normalized to grams of tension and rate of tension development per mm^2. The results obtained during interventions were expressed as % of baseline values.

Major Findings: The experiment is approximately 90% completed. Preliminary results indicate that there is a highly significant age difference in developed tension and maximal rate of tension development in response to the maximal dosage of catecholamines, the youngest group performing better than the oldest group. In contrast, there seems to be no significant age difference in response to paired stimulation and exposure to hypoxia.

Significance to Biomedical Research and the Program of this Institute: To date, there is no available data on the age differences in the response of isolated heart muscle to catecholamines paired stimulation and acute hypoxia. This project will contribute original observations in these areas forming the basis for an overall study of the role of the sympathetic nervous system in the cardiovascular response to stress of the aging animal. It also provides insight into age effects on the general responsiveness of heart muscle to react to inotropic stimuli and acute hypoxia.

Proposed Course of the Project: To complete the remaining 10% of the experiments and to fully analyze the data. In addition to control group, using the highest dose of norepinephrine as the initial dose must be examined; subsequent to this, insight into the mechanisms for the age difference in response to catechols will be sought by determining tissue catecholamines, response after catecholamine depletion, and the response to isoproterenol.

Honors and Awards:

Preliminary results presented at the Research Seminar of the Division of Cardiology at Johns Hopkins University.

Publications: None.

Serial No. HD-AG21(c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Endocrinology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The renin-angiotensin system. I. Chemical-radiosotope derivative assay of plasma renin and angiotensin. II. Plasma renin-plasma aldosterone interrelationships during aging in man. III. Labeled polymeric substrates for renin and related enzymes. IV. Inhibitors of renin: peptides and proteins.

Previous Serial Number: HD-AG21(c)

Principal Investigators: Robert I. Gregerman
Robert J. Workman
J. Howard Lutz (Resigned 9/30/72)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:
Total:
Professional:
Other:

Project Description:

Objectives: Renin is a protease, previously thought to have a high degree of specificity, which is released from the juxtaglomerular apparatus of the kidney. A variety of physiologic and pathophysiologic stimuli are responsible for stimulation or inhibition of renin release. Once in the circulation the enzyme cleaves a decapeptide, angiotensin I, from a circulating plasma globulin. This peptide is activated upon passage through the lung to form an octapeptide, angiotensin II, which pharmacologically is a powerful blood pressure elevating material and physiologically appears to regulate the production of the major adrenal steroid hormone, aldosterone, regulating in turn mineral metabolism. Aldosterone is directly involved in certain forms of hypertension, while the excessive release of renin is directly involved in another type of hypertension, so-called renovascular hypertension.

The chemical measurement of renin has an important place in clinical diagnostic circumstances, while very precise measurement of the enzyme continues to be a major problem area for research. For example, "low renin" hypertension has recently been claimed to be associated with decreased cardiovascular mortality, while a renin concentration ratio greater than 1.5 between the two renal veins is said to herald a successful surgical result

in renovascular hypertension. Physiologic studies in aging man have been very limited, but a major age-related change of aldosterone secretory rate has suggested that altered renin physiology occurs at advanced age.

Because of these considerations, our laboratory has been interested in the development of new approaches to the measurement of renin and angiotensin, in the chemistry of the peptide and the enzyme, and in the physiology of the renin-angiotensin-aldosterone system in normal and pathologic aging.

Methods Employed: Previous reports have given the details of a double isotope derivative assay for measurement of renin through assay of angiotensin I. We have in the past year used this technique to compare renin assays by our own technique and that of others.

Another aspect of this phase of our work uses a labeled polymeric substrate assay for renin which we have developed and previously described. With this technique we have in the past year studied a variety of protein and peptide inhibitors of the enzyme. In addition, a new labeled polymeric substrate has been devised and studied.

Major Findings: I. Using our double isotope assay for angiotensin I we have continued our comparison of plasma renin levels with samples simultaneously assayed by the method of Dr. Anne Gould in her laboratory at the Mt. Sinai Hospital, Cleveland, Ohio. Two new series of samples, in addition to the ones we described in last year's report, were studied. In the two series the correlation coefficients for the two assays were 0.8 and 0.9 However, some individual samples were widely discrepant with the two methods. In the Gould technique, incubation conditions are nearly identical to our own, but the angiotensin I generated is quantitated by bioassay. It is difficult to reconcile these findings and extremely unlikely that the discrepant results are caused by technical errors. The possibility must now be seriously entertained that the angiotensin I generated by certain individuals is chemically and biologically different from the majority of individuals in the population. It would be possible with modification of presently available techniques to determine the amino acid composition of angiotensin I generated by individual human subjects. If significant variations exist, these might have great importance physiologically and in certain hypertensive states.

II. Further work with various immunoassay procedures for angiotensin using a variety of commercial kits as well as antibody and other reagents prepared in our own laboratory have convinced us that all of the methods in current use are unpredictable and,in our hands at least, lacking in precision. As such they are poorly suited to the type of physiologic studies we have previously proposed, namely, correlations of plasma renin activity and concentration with the secretion of aldosterone in normal aging man. Our collaborative efforts with investigators at the Johns Hopkins University have therefore been abandoned for the present.

III. In addition to the previously described synthetic substrate for renin, N-acetyl-poly-L-glutamyl-(M.W.50,000)-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr- Ser labeled with ^{125}I, we have now prepared a new synthetic

substrate. This material was prepared primarily in the hope that it would not be attacked by pseudorenin of plasma. The structure of the new polymeric substrate more closely resembles that of the natural substrate for renin in that its cleavage yields the N-terminal rather than the C-terminal peptide fragment of the synthetic tridecapeptide (or tetradecapeptide) renin substrate. The new material is Arg-Val-Tyr-Ile-His-Leu-Leu-Val-Tyr-Ser-poly-L-Lysine, M.W. 50,000. The poly-L-Lysine is partially acylated by trifluoroacetyl residues. The substrate has been labeled with ^{125}I. Renin cleaves this substrate to yield Arg-Val-Tyr-(^{125}I)-Ile-His-Pro-Phe-His-Leu. This nonapeptide was prepared separately by Edman degradation of angiotensin I and then labeled with ^{125}I.

The cleavage of this new polymeric substrate has been only partially studied. The K_M for the reaction is approximately that of the poly-L-glutamyl substrate (about 0.5×10^{-6}M) but the V_{Max} appears to be considerably less. In addition the cleavage reaction is strongly inhibited by human plasma. Cleavage of the new substrate by plasma has not yet been studied. Although the rather low V_{Max} is not encouraging, the results do already indicate a strong influence of sites remote from the Leu-Leu sequence and suggest that it may well be possible to impart a greater degree of specificity to other synthetic substrates. Several other syntheses of soluble substrates are now under consideration as are further studies on the lysine polymer.

IV. Studies of the inhibition of renin by hemoglobin and other Leu-Leu containing proteins was reported last year. This study has now been greatly extended. The working hypotheses that Leu-Leu sequences account for the inhibition of the enzyme and that renin may attack sequences other than the Leu-Leu sequence of natural or synthetic renin substrate have now been tested.

Hemoglobin chains (horse)were prepared by ion exchange chromatography, cleaved with cyanogen bromide, and two particular peptide fragments isolated, the $\alpha 1$-32 sequence which contains no Leu-Leu sequences, and the β_{1}-55 which contains two. The Leu-Leu sequence was clearly shown not to be an absolute requirement for inhibition of renin. This unexpected finding led us to explore a number of other small synthetic peptides as potential inhibitors of renin. A number of these proved to be quite potent. All contained at least one leucine, but the Leu-Leu sequence was not required. Surprisingly such a simple peptide as Leu-Leu-Leu was effective while Leu-Gly-Leu was not. Leu-Tryp -Leu was also quite potent as was Leu-Tryp-Met-Arg-Phe-Ala. Thus a large number of peptides have now been shown to be inhibitors of renin, and a Leu-Leu sequence is not necessary.

While we were still entertaining the possibility that renin might interact with and cleave Leu-Leu sequences in hemoglobin, i.e., that hemoglobin inhibition was due to Leu-Leu sequences which were acting as alternate substrates, we prepared hemoglobin labeled with 14C-glycine, This material has been shown by others to be a sensitive acid protease substrate. We then showed that renin preparations cleave this labeled hemoglobin. Such preparations have been previously believed to be free of protease activity primarily because they are free of peptidase activity as tested with angiotensin I or II. Thus, we now provide evidence that renin may have a lower degree of

specificity than heretofore suspected. Such a conclusion is in keeping with the relative specificity characteristics of other acid proteases of wide biological distribution. Recognition of renin as an acid protease, similar to other acid proteases in that it is inhibited by pepstatin, a peptide of microbial origin, has been very recently reported, but the specificity of renin has not been previously challenged. Further confirmation of this view will require purification of the enzyme.

Our recent kinetic studies of the inhibition of renin by proteins and peptides have shown that the inhibition is strictly competitive for some inhibitors (e.g. Leu-Leu-Val-Tyr-Methyl ester and lactoglobulin B) but that some contribution by non-competitive factors is involved in the inhibition by others (e.g. hemoglobin and Leu-Trypt-Met-Arg-Phe-Ala). Control experiments established that the inhibition seen with the proteins and peptides was not due to interaction of these materials with the synthetic labeled polymeric substrate. For example, hemoglobin was found to inhibit the action of renin on the natural protein substrate (from hog plasma) with the decreased angiotensin I formation being measured by both radioimmunoassay and by double isotope derivative assay. In addition, it has previously been shown that the peptide Leu-Leu-Val-Tyr-Methyl ester inhibited the action of rabbit renin on rabbit plasma substrate. These observations rule out the possibility that the inhibition seen by the peptides studied here were in some way related to use of the labeled polymeric substrate.

Significance to Bio-Medical Research and the Program of the Institute: Hypertension is an age-related disease of major importance. The enzyme renin is somehow involved in the pathogenesis of this disease. Our own efforts are related to the development of improved diagnostic techniques for measurement of the enzyme, assays for which have assumed a significant role in the differential diagnosis of hypertension, its treatment, and in further research in the area. In addition, our work is relevant to understanding basic aspects of the chemistry of the enzyme renin and of angiotensin related peptides.

Proposed Course of Project: Studies will continue of the peptide inhibitors of human renin. Specifically, new inhibitor studies with pepstatin, leupeptin and diazo compounds will serve to relate renin to the general class of enzymes known as acid proteases. The inhibitor studies will allow choice of suitable compounds for the purification of renin by affinity chromatography. Additional synthetic labeled polymeric substrates will be prepared in an effort to obtain the specificity required for use of these substrates with human plasma. An effort may be made to identify the amino acids of angiotensin I in individual human subjects.

Honors and Awards: Dr. Robert Gregerman was elected to Membership in The American Society for Clinical Investigation at the Annual Meeting of The Society in Atlantic City, N.J., April 30, 1973.

Publications: Bath, N.M. and Gregerman, R.I. Labeled polymeric substrate for renin. Synthesis of N-acetyl-poly (L glutamyl)-[^{125}] tridecapeptide and use for enzyme assay. Biochemistry 11:2845, 1972.

Gregerman, R.I. and Kowatch, M.A.: Isotope derivative assay of nanogram quantities of angiotensin I: Use for renin measurement in plasma. J. Meienhofer (editor), Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium, p.533, Ann Arbor Science Publishers, Inc., 1972.

Serial No. HD-AG22(c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Endocrinology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Relationship of age to thyroid function and thyroid hormone metabolism.

Previous Serial Number: HD-AG22(c)

Principal Investigators: Robert I. Gregerman
Barry S. Handwerger

Other Investigators: Paul J. Davis*

Cooperating Units: Baltimore City Hospitals

Man Years:
Total:
Professional:
Other:

Project Description:

Objectives: This study was a long term project aimed at elucidating the control mechanisms for the metabolism of thyroid hormones. In the recent past the work centered around the role and isolation of the intracellular binding proteins for thyroxine and triiodothyronine.

Methods Employed: A varity of techniques including protein electrophoresis, equilibrium dialysis, etc., have been previously described.

Major Findings: With the resignation of one of the major investigators (Dr. Handwerger) The project was discontinued in this Section, but the work has progressed in the cooperating unit (Dr. Davis) and has been described with their annual report.

Significance to Bio-Medical Research and the Program of the Institute: The project area remains of great interest to Gerontology and to Endocrinology. The role of intracellular binding proteins in the action of thyroid hormones remains to be defined. The mechanism of the age-related slowing of thyroid hormone metabolism is still unknown, and its elucidation would be an important clue to other age-related alterations of hormone and drug metabolism.

Proposed Course of Project: Active work in this area closed within the Endocrinology Section soon after July 1, 1972. Although an informal collaboration with the Endocrinology Division of Baltimore City Hospitals (Dr. Davis) will continue, no further active efforts are planned with the Endocrinolgy Section for the immediate future.

Honors and Awards: None

Publications: None

Serial No. HD-CP 18
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Endocrinology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1,1972 through June 30, 1973

Project Title: Hormone receptors and aging: Effects of age on hormone mediated alterations of the adenyl cyclase, tissue cyclic AMP and factors controlling these metabolic regulators.

Previous Serial Number: New Project.

Principal Investigators: Robert I. Gregerman
Martin J. Kalish

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:
Total:
Professional:
Other:

Project Description:

Objectives: Adenyl cyclase is a membrane-bound enzyme which catalyses the conversion of adenosine triphosphate (ATP) to adenosine-3' -5' cyclic monophosphate (cyclic AMP). The enzyme is very widely distributed in nature. It is well established that in their initiation of effects various hormones activate adenyl cyclase and thus increase the cellular concentration of cyclic AMP. Cyclic AMP then in turn initiates a variety of metabolic effects, depending on the tissue involved. This process is known as the "second messenger" concept of hormone action.

Despite a large and growing literature on the cyclase-cyclic AMP system, no studies are reported which relate to the effect of aging in this area, despite the fact that a number of hormone responses are known to be greatly altered at advanced age. Recently reported studies indicate decreased cyclase activation by several hormones in adipose tissue during maturation, increased phosphodiesterase (an enzyme responsible for cyclic AMP degradation and thus partially for the control of tissue levels of cyclic AMP) and a differential rate of loss of responsiveness depending on the hormone involved. In addition, basal levels of cyclase activity decrease sharply during the first few months (maturation) in the lifespan of the rat.

Our present study aims to investigate effects of aging, and especially of senescence, on hormone mediated activation of adenyl cyclase and the production of intracellular cyclic AMP. The information developed will be useful in determining whether the already known alteration of tissue sensitivity to certain hormones is the result of an age-related alteration of the initial step in hormone action. The study will also allow an examination of the effect of senescence on one aspect of membrane function.

Methods Employed: Adenyl cyclase is being assayed by an established labeled substrate method which allows the determination of ^{32}P-labeled cyclic AMP produced from α-labeled ^{32}P-ATP. Tissue cyclic AMP will be determined by an established competitive protein binding method which is now being standardized in our laboratory. Phosphodiesterase will be assayed by one of several published methods. Rats of both sexes and various ages are being obtained from the animal colony at the Gerontology Research Center. Basal enzyme levels, tissue concentration of cyclic AMP, and phosphodiesterase will be examined in rats of various ages.

Major Findings: Initial results indicate that basal levels of hepatic adenyl cyclase in crude homgenates are not different in one year (mature) and two year old (senescent) rats. However, the response to activation by epinephrine is greatly increased (approximately doubled) in the senescent animals ($p < .05$; $n=6$) for both males and females. Dose response data are available for epinephrine and show that the age difference exists at all concentrations studied to date (10^{-12}M to 10^{-6}M) In contrast the cyclase response to glucagon does not appear to be affected over a wide range of concentrations nor does the activation by the non-specific effect of fluoride ion.

Significance to Bio-Medical Research and the Program of the Institute: These studies should provide an important insight into the mechanisms by which age affects the organism's response to hormones at advanced age. The results already indicate a selective alteration of cyclase responses during senescence. In addition, our studies offer an opportunity to study a membrane bound enzyme and will thus give an insight into age effects on membrane function. The work is directly related to the mission of the Gerontology Research Center and the Institure to provide new information in the area of research on Aging.

Proposed Course of Project: We plan to pursue and to expand our present work. A variety of hormone effects will be studied in a number of tissues. The selection of tissues and hormones is dictated by information already available on altered tissue responses with aging.

Honors and Awards: Dr. Gregerman was an invited Speaker at the 9th International congress of Gerontology, Kiev, USSR, July, 1972. He spoke at an Interdisciplinary Symposium on Hormones and Aging.

Dr. Gregerman was also Co-Chairman of a Section meeting on Hormones at the same Congress.

Dr. Gregerman was invited to contribute a Chapter to the new (5th) Edition of Williams' Textbook of Endocrinology. His Chapter "Hormones and Aging" is the first of its type in a leading standard text on Endocrinology. The Chapter has been submitted and accepted.

Publications: Gregerman R.I., Mechanisms of age related alteration of hormone action and metabolism: Adrenal and thyroid hormones. Proceedings 9th International Congress of Gerontology, 2: 392,1972.

Serial No. HD-CP7 (c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The kinetics of glucose and insulin metabolism in man

Previous Serial Number: Same

Principal Investigators: Jordan Tobin
Reubin Andres
Karl Kramer
Paul Insel

Other Investigators: Ralph DeFronzo
John Rowe
*Mones Berman

Cooperating Units: *National Cancer Institute
Baltimore City Hospitals

Man Years:

Total:	4.1
Professional:	2.2
Other:	1.9

Project Description:

Objectives: These studies are aimed at delineating various parameters of insulin release, distribution, and metabolism in man and the changes in such parameters that occur in altered physiologic states, including aging, diabetes mellitus, and obesity. Because of insulin's role as a prime determinant of glucose utilization, a definition of insulin kinetics will provide a more precise understanding of how the metabolism of glucose is controlled. Current studies are being performed to develop an insulin kinetic model, a glucose kinetic model, and to describe the homeostatic relationship between those two models in normal, diabetic, and obese man of differing ages. From such an analysis, important physiologic controls will be described and clues to pathogenesis of altered metabolic states may be forthcoming.

Methods Employed: The glucose clamp technique was utilized to maintain human subjects at basal arterial blood glucose concentrations after experimental perturbations of the arterial plasma insulin level. The clamp technique employs a controlled variable-rate IV infusion of glucose solution with rapid measurement of blood glucose concentration by an automated method. A negative feed-back formula is applied at 5 minute intervals in

order to servo-correct the glucose infusion rate; thus, the blood glucose concentration is maintained at euglycemic levels. The amount of infused glucose, an index of glucose utilization, provides an estimate of sensitivity to exogenous insulin.

Intravenous infusions of insulin were given by 2 techniques: (1) single shot intravenous injection of .025 crystalline porcine insulin per kg body weight or (2) prime plus continuous infusion at 1 or 2 mU per kg body weight per minute. The prime was given over a 10 minute period such that the total insulin infused in that time was twice the rate infused continuously from 10 to 80 minutes. The infusion during the 10 minute prime was at a logarithmically falling rate which approached the continuous infusion rate asymptotically. The goal of this technique was to create a nearly instantaneous square wave of insulin concentration at a new steady state level.

In some studies glucose-1-^{14}C was injected as a primed continuous infusion during the pre-clamp period to achieve a steady state specific activity and specific activity of glucose (corrected for metabolites and recycled glucose by established procedures) was followed after the administration of insulin. Such radioactive glucose data allows determination of endogenous glucose production and of glucose utilization as a means of analyzing insulin action.

Plasma immunoreactive insulin concentrations, plasma glucose radioactivity, and plasma glucose concentrations were utilized in mathematical modeling of insulin kinetics and glucose kinetics by the SAAM program of Berman and Weiss to determine compartmental dimensions, transfer rates, and to investigate the mechanisms by which insulin regulates glucose utilization.

Major Findings: Work has continued on the computer modeling of glucose and insulin metabolism. The 3 compartments and their exchange rates for glucose and insulin are now defined, and these parameters are being used in a comprehensive analysis of our servo-control glucose-clamp studies:

(1)The development of the glucose model has made it possible to compute moment-to-moment glucose content in the three glucose compartments. Under experimental conditions in which blood glucose concentration is changing, as, for example, in the early part of the hyperglycemic servo-control studies, the infused glucose needed to maintain the desired blood glucose concentration can now be accurately split into two components: (1) the glucose which has simply been added to the (extracellular) glucose spaces and (2) the glucose which has been metabolized. Previous techniques for carrying out these computations had to be based upon the assumption that glucose was distributed in a single compartment. The error involved in the single compartment assumption has now been shown to be very large. The availability of the 3 compartment parameters has added greatly to the accuracy of the estimates of glucose metabolism under these controlled conditions.

(2) These parameters have also enabled us to derive the insulin concentration pattern in interstitial compartments from the complex biphasic plasma insulin curves induced by programmed steady-state hyperglycemia. Thus, we now have better estimates of changes in insulin concentration at the

the site of its action. The metabolic response to the induced insulin responses (i.e. uptake of glucose) is much better related temporally to insulin concentration in compartment 3 (interstitial fluid of peripheral tissues) than to concentration in plasma per se. These results thus further validate the importance of the compartment 3 insulin concentrations; we previously described the controlling effects of this compartment in our euglycemia "insulin - clamp" studies.

(3) The computer modeling of the glucose and insulin compartments of the body has been supplemented by the development of a model of the pancreatic beta cell. The model consists of rapidly and slowly releasing insulin compartments, both fed from a common source. Control of the system can be exerted either at the source or between the source and the releasing pools. The model is able to simulate the complex pattern of pancreatic output of insulin, and by coupling the beta cell model to the 3 compartment insulin model (extrapancreatic) we can simulate plasma insulin responses. These models were able to fit data from both young and old subjects by adjusting the controlling parameters. The ability to define these parameters will provide better understanding of factors which result in altered beta cell function, for example, age, obesity, diabetes, and uremia.

Significance to Bio-Medical Research and the Program of the Institute:
The remarkable prevalence (50%) of abnormal glucose tolerance tests in the older population of the United States coupled with the increased morbidity and mortality of patients with diabetes mellitus demands a delineation of the effects of aging on the pathophysiology of carbohydrate metabolism. These studies in man define kinetic parameters in the glucose-insulin homeostatic system and will apply such analysis to patients with disorders in that system.

Proposed Course of Project: Further refinement of the pancreatic beta cell model will be done. The interaction of obesity and age with insulin and glucose kinetics will be explored. Further ^{14}C-glucose studies will be done to investigate basal glucose turnover as a function of age.

Honors and Awards:
Dr. Andres was an invited speaker at a symposium in Glasgow, Scotland in September 1972 on Recent Advances in Geriatric Medicine.

Dr. Tobin was an invited speaker at an American Geriatric Society Symposium in April 1973 on Geriatric Medicine for the Practicing Physician.

Dr. Rowe was an invited speaker at a Diabetes Seminar at the New York Medical College Department of Medicine in November 1972.

Dr. Andres was an invited member of the faculty at the 4th Summer Biology of Aging Course at the University of Santa Cruz in August 1972.

Dr. Andres was elected as Vice-Chairman of the Clinical Medicine Section of the Gerontological Society.

Dr. Andres was appointed as Chairman-Elect of the Research Committee of the

Gerontological Society.

Dr. Tobin was Visiting Professor of Medicine (Gerontology) at the University of Nebraska, February 1973.

Publications: None

Serial No. HD-AG 10 (c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effect of age on carbohydrate metabolism

Previous Serial Number: HD-AG 10 (c)

Principal Investigators: Reubin Andres
Jordan Tobin

Other Investigators: Karl Kramer
John Rowe

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	3.0
Professional:	0.9
Other:	2.1

Project Description:

Objectives: This project is part of the long-term prospective study of human aging, the Longitudinal Study of the Gerontology Research Center. The complex interplay of aging, carbohydrate metabolism and diabetes mellitus offers a prototype of one of the serious challenges to clinical gerontology, the differentiation of normal aging changes from specific disease states. The objectives of this project are: (1) to describe cross-sectional changes during the entire adult span of life in performance on the commonly-used clinical tests for the diagnosis of diabetes, (2) to provide age-adjusted standards of normality for these tests, (3) to define the relative sensitivity and specificity of these tests, (4) to establish the role of certain experimental variables which influence performance on these tests, (5) to investigate the mechanisms underlying the age changes, and (6) to evaluate the significance of the decline in tolerance [a] by noting the influence of poor performance on mortality and [b] by testing for correlations between performance and certain other variables known to be clearly associated with the overt diabetic state.

Methods Employed: Subjects are primarily those in the Longitudinal Study supplemented by college students and laboratory and hospital employees to increase the sample size of the 3rd and 4th decades. Subjects are given

one of the following tests per visit: oral glucose tolerance (OGTT), intravenous glucose tolerance test (IVGTT), cortisone glucose tolerance (CGTT), or intravenous tolbutamide response (TRT). On the fifth visit the cycle is re-started. Diabetic patients are available primarily from the Baltimore City Hospitals Out-Patient Department; "chemical" diabetes and a small number of overt diabetics are available from the Longitudinal Study Group.

Major Findings: We described previously the development of a "hot box" technique for arterializing venous blood draining the hand. The goal of this technique was to enable us to study participants in the Longitudinal Study with the glucose clamp method but without the necessity of brachial arterial cannulation. The clamp technique provides much more information about pancreatic beta cell responsivity and insulin sensitivity than can be obtained from the routine clinical tests for diabetes. We have studied 28 of the participants in the Longitudinal Study who had characteristics which were needed for the completion of these studies. Our goal has been to have at least 5 subjects in each of 9 categories: 3 age groups (20-39, 40-59, 60-79 years) each with 3 degrees of obesity (obesity index 0.85-1.14, 1.15 - 1.29, and 1.30-1.44). With this grid we should have an adequate description of age differences and of the effect of both mild and moderate obesity. This grid also provides the necessary control data for judging responses of subjects with diseases of interest, especially patients with diabetes mellitus.

This 9 category grid is needed both for the hyperglycemic studies and for the euglycemic insulin clamp studies. The minimum number of total control subjects required is therefore very large (90) and the ability to carry out this complex test on the Longitudinal Participants is an important achievement.

In the past year we have studied 28 of these volunteers: 10 were normal weight controls, 8 were obese but non-diabetic, and 10 were diabetics. The procedure was very well-tolerated, the only complication being an unusual hand-edema which was diagnosed by dermatologic consultants as thermal allergy; it passed off without sequellae in several hours.

A series of 5 studies were done in order to evaluate neuro-endocrine responses to hypoglycemia. It has been proposed that a rapid rate of fall in glucose concentration could stimulate counterregulatory mechanisms even in the absence of hypoglycemia. We therefore created steady-state hyperglycemia (300 mg%) with the glucose-clamp technique and then stopped the glucose infusion. As blood glucose levels fell arterial blood samples were collected for assay of counterregulatory hormones (catecholamines, glucagon, adrenal corticosteroids, and growth hormone). The studies have been completed and endocrine assays are in progress.

Statistical analysis of the routine diagnostic tests for diabetes will be resumed next year. These variables had a lower priority than others in the Longitudinal Study this past year.

Proposed Course of Project: (1) Analyze the routine diagnostic tests for diabetes

through the end of Cycle G (June 30, 1973). These computations in association with the other variables measured in the Longitudinal Study will provide the evidence to meet goals (1) to (4) and goal (6) as outlined in the Objectives section. The interrelated variables are the following: (a) glucose and insulin responses on the clinical tests, (b) serum lipids, (c) body composition, especially lean body mass and obesity, (d) physical activity, (e) dietary factors, and (f) presence of atherosclerotic disease.

Honors and Awards: None

Publications: None

Serial No. HD-CP12(c)
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through July 30, 1973

Project Title: Cytoxan-induced water intolerance

Previous Serial Number: Same

Principal Investigator: Ralph A. DeFronzo

Other Investigators: Paul J. Davis**
Michael Colvin*
Hayden Braine*
Gary Robertson***

Cooperating Units: Baltimore City Hospitals
* Oncology Service
**Endocrine Service
***Indianapolis VA Hospital, Indianapolis, Indiana

Man Years:

Total:	0.20
Professional:	0.20
Other:	0.00

Project Description:

Objectives: High dose cytoxan therapy (30-120 mg/kg/day) has been employed with increasing frequency in organ transplantation and in the treatment of neoplasia. With these high doses a syndrome of weight gain, hyponatremia, and decreased urine output with high urine osmolality has been observed. The goals of this study are to determine the frequency with which the syndrome occurs and to define the underlying mechanism.

Methods Employed: Twenty-two studies were performed in 17 patients. All had normal renal function and excreted a water load normally prior to cytoxan administration. All subjects received 3 liters of normal saline IV for one day preceeding and throughout the period of cytoxan administration (15-100 mg/kg/day). Fluids were permitted ad lib. Urine flow and osmolality and serum sodium and osmolality were followed every four hours; weights were obtained every 6 hours. Because of the possibility of a cytoxan-induced vascular leak, combined ^{95}GRBC volume and ^{131}I albumin space were determined before and during cytoxan administration.

Major Findings: Are summarized in the following table.

	Increase in Uosm (mOsm/L)	Decrease in Urine Flow (ml/hr)	Wt. Gain (kg)	Decrease in Serum Na (mEq/L)	Decrease in Serum Osm (mOsm/L)
CY Responders (N=17)					
Average	514	118	2	9	15
Range	305-798	28-203	0.4-5.6	3-20	6-41

A close temporal and quantitative relationship was found between the excretion of CY active alkylating metabolites (CY-AM) and changes in urine osmolarity. Creatinine clearance and urine sodium excretion remained unchanged (12 studies). AVP levels were normal prior to CY and suppressed physiologically following the decrease in serum osmolality.

Significance to Bio-Medical Research: With low circulating levels of AVP and without change in glomerular filtration or urine sodium excretion we have concluded that CY-AM exerts a vasopressin-like effect on the distal renal tubule. Studies of the effect of CY-AM on the urinary toad bladder are presently in progress. With the development of an in vitro assay system which simulates the effect of CY-AM on the renal tubule hopefully specific therapy can be developed which would prevent the water retention that at times has resulted in congestive heart failure, especially in elderly patients with compromised cardiac function.

Proposed Course of Project: Study has been completed.

Honors and Awards: None

Publications: Water Intoxication in Man Following Cyclophosphamide. Time Course and Relationship to Drug Activation. Ralph A. DeFronzo, H. Braine, O.M. Colvin, P.J. Davis. Accepted for Publication, Annals of Internal Medicine, June 1973.

Serial No. HD-CP 19
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Multiple Myeloma and the Kidney

Previous Serial No: New Project

Principal Investigator: Ralph A. DeFronzo

Other Investigators: Richard L. Humphrey*
John Wright**
J. Robert Cooke***

Cooperating Units: Baltimore City Hospitals
*Oncology Service
**Department of Pathology
Johns Hopkins Hospital
***Renal Division

Man Years:

Total:	0.2
Professional:	0.2
Other:	0.0

Project Description:

Objectives: Renal failure is present in 20-50% of individuals with multiple myeloma on initial presentation and develops in greater than 50% of such individuals at some time during their course. To date the pathogenesis of this renal failure has not been delineated. The classical teaching suggests that obstruction of tubules by myeloma protein is primarily responsible for the deterioration in renal function. However, in several large retrospective studies myeloma casts were not found in up to 15-40% of individuals with severe renal dysfunction. No prospective studies on renal function in myeloma have been reported and consequently the pathogenesis of the renal failure remains largely speculative. This prospective study was undertaken to correlate changes in glomerular and tubular function with renal histopathology and myeloma protein abnormalities.

Methods Employed; 35 patients with multiple myeloma consecutively admitted to the Baltimore City Hospitals Oncology Service were studied. Renal acidification was evaluated with the standard NH_4Cl test (0.1 gm/kg); concentrating ability was examined after a 12 hour overnight dehydration followed by a one hour intravenous infusion of 300 mU of aqueous vasopressin. PAH clearance

were performed to determine renal blood flow. Twenty four hour creatinine clearance was used as an index of GFR. Urine and serum protein was examined both quantitatively and qualitatively (immunoelectrophoresis). After completion of the renal functional study, a renal biopsy was performed. All tissue was examined by routine light microscopy (with amyloid and calcium stains also) electron microscopy, and immunofluorescence.

Major Findings: 13/35 (37%) patients had definitely impaired renal function as defined by a creatinine clearance less than 40 ml/min. Renal tubular function was impaired disproportionately to GFR. Severe acidifying and concentrating defects were noted in many patients with normal creatinine clearance. PAH was also diminished disproportionately to GFR. There was a strong correlation between impaired glomerular and tubular function and the presence of Bence-Jones proteinuria. No patients with balanced immunoglobulin synthesis (i.e. absence of Bence-Jones protein) had a creatinine clearance less than 40 ml/min; 12/23 (52%) patients with demonstrable Bence-Jones protein had Ccr less than 40. Lambda Bence-Jones protein (60%) was more commonly associated with renal function than Kappa (43%).

Renal histologic examination revealed that tubular atrophy and degeneration and not tubular casts correlated best with impaired renal function. Furthermore, only patients with demonstrable Be ce-Jones protein manifested renal structural abnormalities.

Significance to Bio-Medical Research: (1) This prospective study clearly demostrates a close relationship between the presence of Bence-Jones protein and impaired renal function. Furthermore, the marked tubular dysfunction with spared glomerular function suggests that the B-J protein is a direct tubular toxin. Recent studies which show that 98-99% of B-J protein is reabsorbed and catabolized by the renal tubule support our observations. The knowledge that (a) patients with the B-J protein are at risk to develop renal disease and (b) that the presence of impaired renal tubular function is the earliest manifestation of renal dysfunction, allows us to select high risk patients for aggressive chemotherapy early in the course of their disease before severe renal disease has occurred.

(2) Little has been done to support the patient with myeloma who develops renal failure since the renal disease has been felt to be irreversible. However knowledge that such renal disease results from a direct toxic effect of the B-J protein affords new hope for future therapy. If the patient's myeloma can be arrested with appropriate chemotherapy, yet renal failure persists, such patients would be candidates for chronic dialysis and renal transplantation. If the abnormal plasma cells can be eliminated from the marrow and as a consequence the B-J protein is eradicated, then the transplanted kidney would not be at risk to recurrent "myeloma renal disease".

We have recently transplanted one such myeloma patient. This 62 year old white female presented with plasmacytosis B-J proteinemia and proteinuria, lytic bone lesions, and endstage renal failure. She was treated with intermittant high dose cyclophosphamide with healing of her skeletal lesions, disappearance of marrow plasmacytosis, and loss of B-J protein. However, there was no return of renal function. She was supported with twice

weekly hemodialysis for ten months without evidence of recurrent myeloma. She subsequently underwent a cadaveric renal transplant which continues to function well at present, some 4 months posttransplantation. There was no evidence of recurrent myeloma kidney disease on renal biopsy performed 2.5 months post-transplantation.

Proposed Course of Project: The study has been completed; manuscripts are in preparation.

Awards and Honors: None

Publications: None

Serial No. HD-CP 20
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Uremia and Carbohydrate Intolerance

Previous Serial Number: New Project

Principal Investigator: Ralph A. DeFronzo

Other Investigators: Reubin Andres
Jordan Tobin
Karl Kramer
Jack Rowe
Gordon Walker*
Dan Sapir*
Paul Edgar*

Cooperating Units: Johns Hopkins Hospital
* Renal Division

Man Years

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description:

Objectives: Glucose intolerance occurs in greater than 50% of uremic individuals. However, despite numerous investigations the underlying mechanism (s) has not been agreed upon. Before the advent of dialysis and transplantation the finding of abnormal glucose tolerance in patients with chronic renal disease had little bearing on patient management. However, the availability of these newer therapeutic measures has created new significance for uremic carbohydrate intolerance. Where diabetics have been accepted for chronic dialysis or transplantation the incidence of serious complications and mortality has been 4-5 times higher than in non-diabetics. Thus it has become important to distinguish between true diabetes mellitus and uremia-induced glucose intolerance.

Because of the diagnostic and therapeutic implications of carbohydrate intolerance to the patient with chronic renal disease, it was felt important to summarize the large body of literature that has accumulated on the subject of uremia-induced carbohydrate intolerance and to attempt to explain apparent discrepancies reported by different investigators. This

was done and our review paper has been accepted for publication in Medicine. In this paper we propose the hypothesis that glucose intolerance occurs in those individuals who are unable to augment insulin secretion sufficiently to overcome the peripheral antagonism to insulin that exists in uremic individuals. The studies to be reported here are designed to test this hypothesis.

Methods Employed: Carbohydrate tolerance was evaluated in eight chronically uremic subjects accepted into our hemodialysis program employing the servo-control "glucose clamp" technique previously described by us and with standard intravenous and oral glucose tolerance tests.

Major Findings: Beta cell sensitivity was examined by "clamping" the arterial blood glucose concentration at 125 mg% above fasting levels and measuring the plasma immunoreactive insulin (IRI) response. Glucose metabolized, M, equals glucose infusion rate and is a measure of glucose tolerance. In all eight subjects diminished glucose tolerance was observed pre dialysis. To date four subjects have been restudied post-dialysis and all four have demonstrated significant improvement in glucose tolerance. In one of these four subjects the improvement was due to increased pancreatic insulin secretion.

Insulin resistance was evaluated after a continuous 80 min. intravenous infusion of 1 mU insulin per kg body wt per min, hypoglycemia being prevented by a variable-rate infusion of IV glucose. Since arterial glucose concentration is maintained at a constant level, all of the glucose infused must be metabolized and the glucose infusion rate is a measure of sensitivity of tissues to insulin. In 7/8 subjects mild to severe degrees of insulin resistance were noted prior to dialysis. All four subjects studied post-dialysis have demonstrated increase in insulin sensitivity.

In two of the four subjects studied post-dialysis, the improvement in glucose tolerance could not be entirely accounted for by the increased pancreatic beta cell response or by the enhanced insulin sensitivity. This suggests that a third factor may be important in the genesis of uremia-induced glucose intolerance - namely, an inability of the liver to shut off gluconeo-genesis in response to insulin and glucose.

Compared to the glucose clamp technique the IVGTT and OGTT proved to be poor indicators of the degree of glucose intolerance pre-dialysis; furthermore, in 1/4 patients studied post-dialysis was significant improvement noted despite marked improvement in the hyperglycemic clamp.

Significance to Bio-Medical Research: These results substantiate our hypothesis that carbohydrate intolerance in uremia may result from either insulin resistance, diminished insulin secretion, or a combination of these. In addition they suggest that the liver may also play a central role in the genesis of this glucose intolerance. It is clear that with techniques currently available at most renal centers it is impossible to differentiate genetic diabetes mellitus from uremia-induced glucose intolerance. It therefore, seems unwise to recommend that mild elevation of the fasting blood sugar and mild to moderate glucose intolerance on the intravenous or oral glucose tolerance test to be used as criteria to exclude patients from transplant programs.

Proposed Course: The study will be completed by July 1, 1973.

Honors and Awards: None

Publications: Carbohydrate Metabolism in Uremia: A Review. Ralph A DeFronzo, R. Andres, P. Edgar, and W.G. Walker, accepted for publication in Medicine, April 27, 1973.

Serial No. HD-CP 21
1. Gerontology Research Center
2. Clinical Physiology Branch
3. Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The response to solar-simulating radiation as a function of age

Principal Investigator: K.J. Kramer

Previous Serial Number: New Project

Other Investigators: I. Willis
Department of Dermatotology
Johns Hopkins University School of Medicine

Cooperating Units: None

Man Years:

Total:	0.2
Professional:	0.2
Other:	0.0

Project Description:

Objectives; Actinic damage is a precursor to cutaneous malignancy. The normal sunburn response has therefore been a subject of great interest. This response may be influenced by a number of factors including age and skin pigmentation. The particular spectrum causing "sunburn" is from 290-320 nm; longer wavelength ultraviolet radiation (320-400) causes "tanning" and had been felt to have no effect on sunburn or even to protect the skin from damage caused by short UV radiation (290-320 nm). It has however recently been shown (Willis) that the longer wavelength UV radiation actually enchances damage caused by the 290-320 radiation; this has been termed the photoaugmentative effect. In light of these new findings, coupled with the fact that older studies suffered from serious technical defects, the human sunburn response deserves re-evaluation. Specific objectives are:

1) To determine susceptibility to sunburn as a function of age
2) To determine the photoaugmentative effect of long UV wavelength light in relation to age
3) To determine the time course of the sunburn response of individuals in relation to age
4) To determine all of the above with respect to exposed and non-exposed skin

Methods Employed: Longitudinal participants will be divided into decade age groups and into clinically determined skin pigmentation groups. A total of about 100 subjects will be tested. Individual areas of skin (6mm diameter each - total of 12 sites) on the arm and back will be exposed to increased levels of full spectrum radiation (290-700 nm) (4 sites); short UV irradiation (290-320) (4 sites) and short and long UV radiation (290-400). Exposures will be chosen above and below the minimal amount of energy that will be anticipated to produce erythema over the entire test site (minimal erythema dose or MED). Responses will be read immediately and at 6,8,12, 24,36, and 48 hours by either the investigators or nurses trained for the task.

Results; Preliminary results are confirming the photoaugmentative effect. Somewhat unexpectedly, the immediate response does appear to anticipate the 12 and 24 hour responses even though the physiologic bases for these responses are different. This fact has potential clinical significance in that it could facilitate the time-consuming process of testing and would make screening for abnormal responses practical.

Significance of Results: Confirmation of the photaugmentative effect will change the criteria for sunscreens designed to be prophylactic for the development of cutaneous carcinoma, since present preparations screen primarily short UV light.

In addition, delineation of the evolution of the normal response to solar radiation with age will add new information concerning the aging process in an organ little studied, the skin.

Proposed Course of Project: It is anticipated that the project will be completed by July.

Honors and Awards: None

Publications: None

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Gerontology Research Center
Laboratory of Cellular and Comparative Physiology

FY'73 has been highlighted by a major change in the emphasis of program goals and in the accompanying research activities to achieve these goals.

The current goals are three-fold: (a) to conduct studies on the nature of age-related deteriorating functional changes of cells and tissue systems whose growth and differentiation events are reasonably well understood, (b) to determine the underlying mechanisms of these changes, and (c) to develop methods for early detection of the deterioration and, hopefully, methods to control or reverse the deterioration.

To implement these goals emphasis will be on the use of cells and tissues of mammalian species, including man, in in vitro and in vivo cultures. Moreover only a few closely related model systems will be analyzed, but they will be studied systematically.

The major thrust in research activity has been in immunology and aging, with the creation of the Immunology Section, for the following reasons. (1) Associated with, or as a consequence of immunosenescence, the incidence of immunodeficiency diseases including autoimmunity, cancer and infection increases. (2) Cellular, molecular and genetic knowledge of the ontogeny, phylogeny and differentiation process involved with the immune system is well understood, probably more so than any other functional system. (3) The system is amenable to elegant cellular and molecular analyses, and, therefore, offers great promise for successful manipulation of the system.

During the past year, members of the Immunology Section expanded their effort in the creation of the laboratories and initiation of research projects. Hence fruition of their current research task should become apparent at the earliest during the next fiscal year. Nevertheless, basic findings were obtained in FY'73, some of which were established just prior to their arrival at the GRC, and they are as follows.

(1) Of the three cell types (thymus-derived T cells, bone marrow-derived B cells and accessory A cells) involved in the initiation of a humoral immune response, the T and B cells are altered in immunologically deficient old mice, but A cells appear normal.

(2) The proliferative capacity of T and B cells is decreased as much as 10-fold with age. This is a very critical function because the number of functional effector cells generated in an immune response is dependent upon these cells to divide efficiently.

(3) Limiting dilution, reconstitution, and young cell-old cell mixture analyses bear out indirect evidence that either the relative number or the efficiency of regulator cells that suppress an immune response increases with age. For example, it was found that the humoral immune activity of a mixture

of spleen cells from young and old mice was lower than the sum of individual responses, indicating that there are cells in old mice that can inhibit young immunocompetent cells from responding to antigenic stimulation.

(4) One of the deficiencies of radiation-induced allogeneic bone marrow chimeras was shown to reside in the thymus gland, the seat of immunodeficiency diseases in most cases. Previously it was shown that these chimeras are suitable model animals to study immunosenescence.

(5) Limiting numbers of lymphoid cells undergo maximum antibody response in vitro only when they are cultured under optimum cell concentration, a variable which was not taken into consideration previously. This information offers us the option of assessing quantitatively the activity of limiting numbers of lymphoid cells and, hopefully, one or two clones of competent cells.

(6) Mice with an exceptionally long life span show a decline with age in cell-mediated immune activity and resistance to allogeneic tumor cells. Previously the decline in activity was observed in mice with short life spans. Therefore critics have argued that life-shortening diseases imposing on the immune system were responsible for the decline in cell-mediated immunity. These results should minimize such criticisms.

Based on these findings efforts are focused currently on the cellular and molecular definitions of immunosenescence with emphasis on (a) isolation and characterization of promotor and suppressor cells, (b) ability of stem cells to generate thymus-derived T and bone marrow-derived B cells, (c) life spans of memory cells, (d) mitogen receptor sites and the cyclic AMP system of proliferating T and B precursor cells, and (e) characterization and repair of induced and naturally occurring immunodeficiency states.

Research has also been initiated on a small scale in biomembrane physiology, with emphasis on the effect of age on hormone bindings, for two reasons: (a) it will complement ongoing molecular studies in immunology and (b) the time is "ripe" to initiate cellular and molecular studies focused on the nature of inefficient response to hormones and drugs by the elders. Preliminary results showed that adipose tissue, skeletal muscle, brain and prostate gland of aging rats exhibit a progressive decline in steroid hormone binding due, in part, to a reduction in the number of macromolecular binding sites per unit tissue mass.

Another area of research that can contribute to the program goals of LCCP is mammalian genetics. An understanding of the genetic mechanisms of age-related functional deterioration at the molecular level is obligatory. To this end, research is being promoted to understand, at the cellular level, human and animal disorders manifesting altered development and aging. Currently a laboratory is being created to carry out two studies: (a) the role of chromosomal aneusomy in the impaired proliferation of fibroblasts and immunocompetent cells in relation to aging and (b) the influence of gene dosage and maternal environment on the life span of embryos and individuals carrying lethal genes. The long-term goal of these studies is to "sort out" and elucidate those genetic alterations which can impair the proliferative

capacity of somatic cells. Information derived from this program should have a tremendous impact on cellular biology of aging, for impairment with age of the proliferative capacity is characteristic of certain somatic cells.

Two sections in existence prior to FY'73 are the Nutrition and Morphology Sections. The former is concerned with the influence of nutrition and antimetabolites on the life span and various tissue enzyme activities in rats. The latter is concerned with structural and cytochemical changes associated with development and aging of myocardial and skeletogenic cells and with the growth patterns of coelenterates as influenced by temperature and endogenous rhythmic changes.

During the past year, the Nutrition Section completed preliminary studies in two areas: (a) the effect of cycloheximide on the rate of development of chick embryos and (b) the effect of protein undernourishment on the life span of middle-aged rats. In the former study it was found that nontoxic levels of cycloheximide, an inhibitor of protein synthesis, when given to 1 day old chick embryos delay their rate of development at least until day 17, as judged by morphological parameters, heart beat and DNA content. However, the activities of lysosomal and mitochondrial enzymes were not affected.

The latter study revealed that reduction in the dietary protein intake in 16 month old rats from the standard 24% to 8 or 4% had no influence on their subsequent mean life expectancy. However rats restricted to 12% casein diet lived on the average of 2 months longer. The life promoting effect of 12% protein intake was manifested during the first 3-4 months following dietary restraint. In view of the profound implication this observation offers, a more systematic study is being initiated.

Ultrastructural cytochemical studies by the Morphology Section relating lysosomal activities and aging processes in the left ventricular cells of aging rats suggested that in mural myocardial cells there are (a) two different pathways by which primary lysosomes are formed and (b) two different mechanisms of lysosomal degradation of mitochondria. The two pathways of synthesis, transport and packaging of primary lysosomes are the nuclear pole zone route and the peripheral route. The latter, which bypasses the Golgi, provides the cells a more direct route to sites of action of the lysosomes. Lysosomal degradation of mitochondria occurs by the more common matrix densification process and the less common matrix clarification process. Studies on papillary myocardial cells of 24 month old rats showed that there are two types of changes: (a) cells that appear grossly normal in structural appearance but showing marked intracellular changes in comparison to those of 6 and 12 month old rats, and (b) cells that appear grossly abnormal in structural appearance. Ultrastructural definition of age related deterioration of heart muscle cells may provide us a better insight how to resolve whether the changes observed are the cause or consequence of senescence of the organ.

Studies on endogenous circannual rhythms in growth, development and life spans in marine coelenterates revealed that they can persist for over 3 years in the absence of periodic signals from the environment. The period for the

cycles at three ambient temperatures, 10°, 17° and 24°C, was about one year. The life span of hydranths during prolific growth of the colony was considerably longer than during repressed growth. These observations suggest that there can be exceptions in certain invertebrates to the two widely held views on life span: (a) the span of life of a species is specific and (b) delay of growth can extend life expectancy.

Serial No.: HD-CCP-12
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characterization of Age-related Defect in the Proliferative Capacity of Lymphocytes

Previous Serial Number: None

Principal Investigator: Takashi Makinodan
Margaret L. Heidrick
Other Investigator: None

Cooperating Units: Oak Ridge National Laboratory
Oak Ridge, Tennessee

Man Years:

Total:	1.2
Professional:	0.7
Other:	0.5

Project Description:

Objectives: The proliferative capacity of stimulated lymphocytes is known to be defective in old mice. Both thymus-derived (T) and bone marrow-derived (B) cells are affected as determined by a decrease in the number of antibody-forming progeny generated per immunocompetent unit, a decrease in the mixed lymphocyte reaction and a depressed response to specific mitogens. The objective of this study is to determine the underlying cause(s) of this age-related defect. Three possible causes are being investigated: (1) an increase of regulator or repressor T-cells, (2) a decrease in the number of mitogen receptor sites and/or (3) a defect in the cyclic-AMP system.

Methods Employed: Stimulation of lymphocyte proliferation with specific mitogens is being used as the model system. Spleen cells are separated into distinct populations by size and/or density. The component cells are then assessed for (1) their proliferative ability, (2) number of mitogen receptor sites and (3) changes in their cyclic-AMP system.

Major Findings:

Presence of Repressor Cells. Recent reports in the literature indicate a sub-population of T-lymphocytes may suppress the response of immunocompetent cells and thereby regulate the magnitude of the response. It is thus possible that an increase of such repressor cells could be a causative factor in the depressed proliferative capacity of lymphocytes from old mice.

A search for such suppressor cells in the spleens of old mice has yielded the following information: (1) While the total number of lymphoid cells remain relatively constant with age, the proportion of lighter cells increases. (2) Light and heavy cells can be distinguished by their responsiveness to mitogens, the lighter cells being responsive to pokeweed mitogen and the heavier cells to concanavalin A. (3) The responsiveness of the heavier T cells to concanavalin A can be decreased by mixing them with lighter cells, indicating that there are regulator cells in the light cell fraction that can suppress the heavier cells from responding to the mitogen.

Mitogen Receptor Sites. It is known that specific mitogens bind to the surface of lymphocytes and stimulate the cells to divide by some unknown event occurring at the cell membrane. A possible explanation for the decreased response of aged lymphocytes would be a decrease in the number of mitogen binding sites. To investigate this possibility, the amount of ^{125}I-labeled concanavalin A bound by young and old spleen cells was measured. Preliminary results indicate that both young and old cells bind the same amount of mitogen and suggests that on the average the number of mitogen binding sites on the lymphocyte surface does not decrease with age.

Cyclic-AMP System. The earliest known biochemical event which occurs in lymphocytes after stimulation with mitogens is a sharp rise and then fall in the cyclic-AMP and cyclic-GMP levels of the cells. These changes occur within minutes after mitogen stimulation and there is evidence that changes in the intracellular levels of the cyclic nucleotides initiate and regulate cell proliferation. It is thus possible that a defect in the cyclic-AMP system of the old lymphocytes could be a contributing factor to their defective proliferative capacity. To investigate this possibility, the cyclic-AMP and cyclic-GMP changes in young and old lymphocytes after mitogen stimulation are being measured. This study has just recently been started. To date, most of the preliminary experiments (i.e., determining optimum mitogen concentrations, cell numbers required for obtaining cyclic nucleotide levels, etc.) have been completed but comparative data of young and old cells is not yet available.

Significance to Biomedical Research and the Program of the Institute: Precursor T and B cells generate effector progenies efficiently through proliferation. Therefore reduction in the proliferative capacity of aged precursor cells is an important factor in the reduced immune response of the aged. Correction of this deficiency should have a beneficial effect on the immune system of the aged which, in turn, may lower the incidence of immunodeficiency diseases.

Proposed Course: If the cause or causes of the defective proliferative capacity of the old lymphocytes can be determined, then attempts will be made to correct the defect (i.e., removal of repressor cells, treatment of the cells with various agents to alter the cyclic nucleotide levels). If a correction is achieved, it will then be determined if the ability of T and B precursor cells to function in an immune response is improved.

Honors and Awards:

Dr. Makinodan was invited to present a seminar at the Aging Research Center, Korman Research Pavillion, Albert Einstein Medical Center, Philadelphia, Pennsylvania on December 4, 1972.

Dr. Makinodan was invited to chair a Session on Immunobiology and to present a paper at the 25th Annual Scientific Meeting of the Gerontological Society in San Juan, Puerto Rico, December 17-21, 1972.

Dr. Makinodan was invited to give a seminar at the Dental Research Center, University of North Carolina, Chapel Hill, North Carolina on February 19, 1973.

Dr. Makinodan was invited to present a seminar at the Department of Laboratory Medicine, Medical School, University of Minnesota, Minneapolis, Minnesota on March 30, 1973.

Dr. Makinodan was invited to present a seminar at the Department of Microbiology, University of Vermont, Burlington, Vermont on April 2, 1973.

Dr. Makinodan was invited to present a lecture series at the Department of Microbiology, School of Medicine, Johns Hopkins University, Baltimore, Maryland on May 2-3, 1973.

Publications: Heidrick, M.L., and Makinodan, T.: Nature of cellular deficiencies in age-related decline of the immune system. Gerontologia. In press.

Price, G.B., and Makinodan, T.: Aging: Alteration of DNA-protein information. Gerontologia. In press.

Serial No.: HD-CCP-13

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Characterization of Cellular Deficiencies in Age-related Decline of the Humoral Immune System

Previous Serial Number: None

Principal Investigators: Margaret L. Heidrick
Katsuiku Hirokawa

Other Investigator: T. Makinodan

Cooperating Units: Oak Ridge National Laboratory
Oak Ridge, Tennessee

Man Years:

Total:	1.6
Professional:	1.1
Other:	.5

Project Description:

Objectives: The humoral immune activity of $BC3F_1$ mice declines with advancing age. The cause for the decline is due primarily to defects in the immunocompetent cells. Since three different cell types [accessory cells, thymus-derived lymphocytes (T-cells), and bone marrow-derived lymphocytes (B-cells)] are known to be involved in producing an immune response, the defect in the old mice could be due to a decrease in the number or function of one or more of these three essential cell types. The objectives of this project are to (1) separate the three cell types from diseased and nondiseased spleens of old mice, and (2) determine the functional capacity of each cell type in the old mice.

Methods Employed: Several cell separation techniques are being used in this study in an attempt to separate the different cell types. These include: separation by differential adherence to plastic, differential centrifugation in a discontinuous Ficoll gradient, separation on glass bead columns and separation by size on a Stay-Put cell separator. The purity of the separated cell fractions is being assessed by use of specific anti-sera, specific mitogens, size, and functional capacity. The ability of the old cell fractions to function is being evaluated by combining the old cell with young cell fractions and assessing the humoral response of the various combinations to sheep red blood cell antigen (SRBC).

Major Findings:

Activity of the Accessory Cell. Spleen cell suspensions from young and old mice were separated into two populations on the basis of their ability to adhere to plastic Petri dishes and their activity was assessed in various combinations with SRBC. The accessory cell is present in the adherent population while the T- and B-cells are in the non-adherent population. Old adherent cells cultured with young non-adherent cells responded fully to the antigen, whereas old non-adherent cells combined with young adherent cells gave a response comparable to unseparated old spleen cells. Similar results were obtained when adherent cells were exposed to SRBC, washed free of the antigen, then recombined with the nonadherent cells. The results demonstrate that the adherent cells of old spleens are indistinguishable from those of young spleens in their ability to initiate antibody response, but the activity of nonadherent cells of old spleens is impaired. To further evaluate the activity of the accessory cell, which is thought to be a macrophage, peritoneal macrophages from young and old mice were compared as to (1) number of SRBC engulfed, (2) time required to digest engulfed cells and (3) activity of three lysosomal enzymes--cathepsin D, beta-glucuronidase and acid phosphatase. The results indicated the old macrophages had slightly higher levels of all three enzymes and were equivalent to young macrophages in their ability to engulf and digest SRBC. Thus by various indices it appears that macrophages do not deteriorate with age.

Activity of T and B Cells. In the Ficoll gradient, non-adherent cells separate into two fractions, a light fraction enriched in B-cells and a dense fraction enriched in T-cells. Combining these fractions in various combinations and proportions *in vitro* revealed that an optimal ratio of T and B cells was required to generate a maximum response to SRBC. For both young and old cells, the optimal ratio was about the same. When old spleen cells were separated in the Ficoll gradient, a different separation profile was observed. The old spleens contain an increased percentage of cells separating in the lighter fractions and a decrease in the percentage of cells in the dense fraction. To determine if the altered ratio of T to B cells in the old mice was a contributing factor to the defective response, old spleen cells were separated in the Ficoll gradient and then reconstituted in the ratios observed in young mice. However, our preliminary attempts to improve the response of the old cells by changing the ratio of the Ficoll fractions have thus far been unsuccessful. Replacing individual fractions with young cells was not effective until both fractions were replaced with young cells. Our results indicate both T and B cells are defective in the old mice and that the defect is not due merely to a shift in the ratio of cell types present.

Cell Interaction. To investigate possible deficiencies in the necessary cellular interactions required for an immune response, we have studied the ability of young spleen cells to supplement the defective old spleen cells. For these studies, dispersed pooled spleen cells from young (3 months) and old (24 and 30 months) mice were assessed individually in 1:1 mixtures for their antisheep RBC responsiveness. The response of old spleen cells is always lower than that of young spleen cells. However, the magnitude of response of the mixtures was dependent upon the disease status of the

spleen of old mice and the type of culture used in the assay. In the case of mixtures of cells from old spleens with frank reticulum cell sarcoma, the response in vitro and in vivo was either equal to (additive) or less than (inhibitory), but never greater than (synergistic), the sum of the individual responses. This would suggest that old tumorous spleen cells can inhibit the response of young spleen cells. In the case of mixtures of cells from old spleens free of frank tumors, the response was influenced in part by the type of culture. Synergistic, additive, and inhibitory responses were observed when the mixtures were cultured in vitro and in vivo in diffusion chambers. The synergistic response would suggest that the cells or factors which are limiting in the young spleen cell suspension are in excess in the old spleen cell suspension and vice versa. Interestingly, inhibitory responses were not observed when the mixtures were assessed in vivo in the spleen of irradiated syngeneic young recipients. This would suggest that irradiated young spleens may possess factors that can counteract the inhibitory action of certain old spleen cells from donors free of frank lymphoreticular diseases.

Significance to Biomedical Research and the Program of the Institute: The decline of the immune system with age has an obvious effect upon general senescence and longevity. If the cell type or types which are responsible for the decline can be separated and identified, this will be a significant step toward determining the cause for the decline at the molecular level.

Proposed Course: Further studies will continue to separate and identify the cell types responsible for the decline of the immune system. When this is accomplished, studies will be initiated to determine the nature of the defect in the cells.

Honors and Awards:

Dr. Makinodan served as a member of the Workshop Panel of the Task Force on Immunology and Disease sponsored by NIAID.

Dr. Makinodan was invited to present a lecture on Immunology and Aging by Dr. R. A. Good, Department of Pathology, University of Minnesota, Minneapolis, Minnesota on November 14, 1972.

Dr. Makinodan was invited to present a seminar at the Jefferson Medical College, Department of Biochemistry, Philadelphia, Pennsylvania on November 29, 1972.

Dr. Makinodan was invited to attend and present a seminar on "Cellular Aspects of Age-Associated Impairment of the Immune System" at the Gordon Research Conference, Biochemistry of Aging, held 1/22-1/26/73 at Santa Barbara, California.

Dr. Makinodan served as a member of the Public Policy Committee of the Gerontological Society.

Dr. Makinodan was invited to present a seminar at the Department of Biology, University of Puerto Rico, Rio Piedras, Puerto Rico on December 22, 1972.

Dr. Makinodan served as Associate Editor of the following journals: Journal of Cellular Physiology, Blood, Mechanisms in Aging and Development, and Journal of Immunology.

Publications: Quinn, R.P., Price, G.B., Ellis, J.M., and Makinodan, T.: Catabolic half-lives of immunoglobulin and albumin as a function of age in mice. J. Gerontol. In press.

Makinodan, T., Heidrick, M.L., and Nordin, A.A.: Immunodeficiency and autoimmunity in aging. In Second International Workshop on Primary Immunodeficiencies in Diseases in Man. National Fdn. Press, Washington, D.C., 1973. In press.

Serial No. HD-CCP-14

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cellular Basis of Regulation of the Humoral Immune Response

Previous Serial Number: None

Principal Investigator: Albert A. Nordin

Other Investigators: Barbara E. Loughman
John J. Farrar

Cooperating Units:

Man years:

Total:	2.3
Professional:	1.6
Others:	0.7

Project Description:

Objectives: The goal of this project is to characterize the regulation of the immune response as expressed by cellular elements. Efforts to determine the origin and mechanism of action of these cells are of prime interest.

Methods Employed:

1) B-cell source-Young C57Bl/6 mice (6 weeks of age) are thymectomized, irradiated 2 weeks later and grafted with 10 x 10^6 syngeneic bone marrow cells 24 hours after irradiation. Four to eight weeks later the spleens of these mice serve as a source of bone marrow derived lymphocytes (B-cells).

2) T-cell source-six to eight week old C57Bl/6 or $B_6D_2F_1$ mice are used as donors for thymus glands. X-irradiated (850r) syngeneic mice are injected with 50-70 x 10^6 thymus cells with or with a simultaneous injection of antigen. Seven or eight days later the spleens of these mice are used as a source of thymus derived lymphocytes (T-cells). Those cells from mice injected with thymocytes and antigen are referred to as activated T-cells and those from mice receiving only thymocytes are referred to as normal T-Cells.

3) The *in vitro* culture technique and the assay for plaque-forming cells are routine methods.

Major Findings:

1) Activated T-cells partially reconstitute the *in vitro* response of B-cells. The ratio of T:B-cells is a critical parameter.

2) A high ratio of activated T:B-cells results in significant suppression of the *in vitro* response.

3) Significant numbers of B-cells contaminate adherent cell layers. Expression of these B-cells requires the addition of activated T-cells.

4) Activated T-cells suppress the *in vitro* response of normal spleen cells.

Significance to Biomedical Research and the Program of the Institute: This proposal attempts to offer two main significant contributions. The basic cellular interactions involved in the total immune response extends into many areas of research. Of great interest in cell biology is the control of cellular functions. The regulation of the humoral immune response by direct cellular interactions and/or by products of "regulator" lymphoid cells is one of the main contributions to be derived from this research. Of equal significance is to demonstrate the role, if any, of this cellular regulation in aging. Any logical approaches to maintenance of a functional immune capacity in aging individuals can only be undertaken when the facts surrounding the problem are firmly documented.

Proposed Course:

1) Investigate the occurrence and distribution of suppressor T-cells. The preparation of normal T-cells as well as determination of naturally occurring suppressor cells in normal spleen cell populations is planned.

2) Identification and characterization of suppressor cells. Physical means of separation, i.e., discontinuously Ficoll gradients, gravity sedimentation, electrophoretic mobility as well as biological methods of preparation, i.e., cell transfer, antisera treatment will be used to obtain an enriched cell fraction for suppressor cell studies.

3) Determine if suppressor cells also function in T-independent immune system. Some success has already been observed using detoxified endotoxin of *E. coli* as a T-independent antigen.

4) Investigate the mechanism of suppression. It is necessary to establish if suppression is exerted by direct cellular interaction or by soluble cell products.

5) The immunosenescence observed in aged mice will be examined by these established methods to determine what role suppressor cells play in the decline of the humoral immune response seen with increasing age.

Honors and Awards: Dr. Albert A. Nordin participated as a visiting staff member in the Cell Culture Procedures for Aging Research course conducted at the University of Vermont on September 22, 1973.

Dr. Albert A. Nordin is presently a member of the Council of the Regulatory Biology Section of the National Science Foundation.

Publications: Nordin, A.A., and Makinodan, T.: Symposium on immunopathology of aging: Humoral immunity in aging. Fed. Proc. In press.

Serial No. HD-CCP-15
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cellular Aspects of the Immune Response of Long-term Radiation Induced Allogeneic Chimeras

Previous Serial Number: None

Principal Investigators: Barbara E. Loughman

Other Investigators: Albert A. Nordin

Cooperating Units: None

Man Years:

Total:	0.3
Professional:	0.2
Other:	0.1

Project Description:

Objectives: The objective of the proposed research is to delineate the immune system of x-irradiated, allogeneic bone marrow grafted mice in an attempt to use these mice as a model for investigating basic cellular aspects of the immune mechanisms and immunodeficiencies.

Methods Employed: Previous work has established that allogeneic bone marrow chimeric (ABMC) mice can only survive in a controlled environment. This environment has previously been the germ free state but there is reasonable evidence to suggest that a clean environment would suffice. This clean environment will be created by using a low velocity laminar-flow tent.

Major Findings: The findings listed below are relevant to this project even though they were obtained prior to instituting this activity at the GRC.

1) ABMC mice show a normal life-span but the humoral immune response is drastically reduced.

2) The lymphoid system of the ABMC mice is completely derived from donor bone marrow cells.

3) The marked humoral immune deficiency has been associated with thymus gland malfunction.

4) The thymic deficiency can be corrected completely in vitro but as yet in vivo reconstitution has failed.

Significance to Biomedical Research and Program of the Institute: The results of these investigations will be significant in a practical sense. Clinical bone marrow grafting within a heterogeneous population has necessarily been restricted mainly as a result of immunologic damage to the host. For obvious reasons, the parameters involved in these reactions are not as easily approached in human as they are in a representative animal model. Techniques are available to experimentally determine not only the favorable conditions, which permit the establishing of allogeneic bone marrow chimeric mice, but also to define the events resulting in immunoregulation observed in such animals. Hopefully, these results will bear a semblance to those encountered in human bone marrow grafting. Information may then be available which will reduce the risks in bone marrow grafting and result in the reconstitution of an intact immune mechanism.

Basic information pertinent to the immune response is equally significant. Cellular immunology, during recent years, revolved around cell cooperation as a basic requisite for the expression of antibody to many antigens. Although the majority of such studies have considered the interactions between thymus dependent and bone marrow dependent cells, it is becoming increasingly obvious that other cell type cooperative efforts are involved in the complex immune systems of mammals. The deficiency of any one cell type most likely results in regulating the expression of immunity involving several lymphoid elements. The information which can be supplied in attempts to re-establish immunological competence to the allogeneic chimeric mice is expected to demonstrate and define the importance of cellular interactions in the both humoral and cell- mediated forms of the immune response.

Studies concerned with immunodeficiency diseases will be of significance since many naturally occuring examples of such diseases have traced a causative factor(s) to the thymus gland. This model system with a well defined thymic defect should provide meaningful data relevant to the immunodeficiency disease state.

Proposed Course:
The specific aims of the proposed research are:
1) To establish long-term allogeneic radiation chimeras in a controlled laminar-air-flow environment.

2) To characterize the humoral and cell-mediated immune responses of the allogeneic radiation chimeras prepared and maintained (at least 4 months) in a laminar flow environment.

3) To locate and define, at the cellular level, the defects(s) involved in the severe regulation of the total immune response of allogeneic chimeric muce.

4) To attempt to relieve this regulation by manipulating the intact animal.

5) To investigate the possible reasons for the more normal life span afforded the allogeneic chimeras prepared in a controlled environment.

Honors and Awards:

1) Dr. Barbara E. Loughman was invited to present a seminar at the Trudeau Institute, Saranac Lake, N. Y., August 30, 1972.

2) Dr. Albert A. Nordin presented a paper at the 25th Annual Scientific Meeting of the Gerontological Society held in Puerto Rico on December 18, 1972.

3) Dr. Albert A. Nordin presented a seminar related to this project on January 10, 1973 to the Immunology Unit at Veterans Hospital, San Francisco, California.

4) Drs. Barbara E. Loughman and Albert A. Nordin presented a seminar related to this project to the Johns Hopkins Immunology Group on January 30, 1973.

Publications:

1) Loughman, B.E., Nordin, A.A., and Bealmear, P.: Studies of the immunological capacity of germfree mouse radiation chimeras. I. Chimerism and humoral immune response. Cell. Immunol. In press.

2) Loughman, B.E. and Nordin, A.A.: Studies of the immunological capacity of germfree mouse radiation chimeras. II. Thymus cell function in a humoral immune response to sheep erythocytes. Cell. Immunol. In press.

Serial No.: HD-CCP-16

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: In Vitro Studies on the Capacity of Aged Mice to Develop Immunological Memory

Previous Serial Number: None

Principal Investigator: John J. Farrar

Other Investigators: Barbara E. Loughman
T. Makinodan
Albert A. Nordin

Cooperating Units: None

Man Years:

Total:	1.5
Professional:	1.3
Other:	0.2

Project Description:

Objectives: The objective of this research is to define the specific memory cell deficiencies in the aged mouse.

Methods Employed: Modifications of the in vitro immunization procedure as described by Mishell and Dutton were used throughout the course of the research. The system involves culturing mouse spleen cells with antigen (sheep erythrocytes) in tissue culture for five days. The generation of antibody-forming cells (AFC) has been assessed by the Jerne and Nordin hemolytic plaque-forming cell assay. In several of the experiments, the spleen cells were separated into non-adherent (lymphoid) and adherent (non-lymphoid) cell populations prior to immunization in vitro. In several experiments, the capacity of bone marrow from aged mice to generate bone marrow-derived (B) lymphocytes has been assessed. These experiments were performed using the in vivo cell transfer system of Claman, Chaperon and Triplett.

Major Findings: Spleen cell dose-response experiments showed that the optimal cell density for development of AFC in vitro is the same for both the young and aged mouse cells. Using this cell density (10×10^6 cells per 1 ml culture), a series of experiments were performed to determine the ability of aged mice to develop immunological memory. Groups of young and

aged mice were immunized intravenously with sub-optimal, optimal, and supra-optimal doses of antigen. At various time intervals thereafter the spleen cells were placed in tissue culture and rechallenged with the same antigen. In each case the secondary direct AFC response of the aged spleen cells was found to be enhanced above the level of the primary in vitro response. However, the secondary responses of the aged spleen cells were still severely deficient in comparison to the respective young spleen cell secondary responses. The number of indirect (IgG) AFC generated was not significant even in those cultures containing young primed spleen cells.

Preliminary experiments on an assay system for assessing the degree of deficiency in the B-lymphocyte memory cell population have been completed. The system involved co-cultivating varying numbers of antigen-"activated" thymus-derived lymphocytes (ATC) with either unseparated spleen cells, non-adherent spleen cells, or anti-θ treated non-adherent spleen cells. In each case the ATC enhanced the response of the aged mouse B-cells but their response still remained deficient in comparison to the young spleen cell controls. The results suggest that aged mouse B-cells are deficient in their ability to generate AFC even when provided with a "purified" and "optimal" source of thymus-derived lymphocytes (T-cells).

The results of the first experiments using the in vivo transfer system suggested that aged bone marrow contained a significant thymus-derived lymphocyte (T-cell) contamination, which was not detected in young marrow. These observations led to the use of anti-mouse lymphocyte serum (ALS) and anti-θ to reduce the T-cell contamination. Subsequent experiments indicated that (1) bone marrow cells from ALS treated aged donors were deficient in their ability to generate AFC, and (2) anti-θ treatment of the bone marrow cells significantly reduced the T-cell contamination of the old marrow but did not depress its capacity to generate AFC. These results suggested that along with the T-cell contamination the aged marrow also contained an additional differentiated population. These cells apparently had an enhanced capacity to generate AFC, suggestive of a peripheral B-lymphocyte. In order to examine this possibility more directly, immunofluorescent studies using labelled antibody against mouse immunoglobulin were carried out. The results revealed an approximately 7-fold increase in the frequency of immunoglobulin-bearing cells in the bone marrow population of aged mice. B-lymphocytes are characterized by the presence of immunoglobulin on their surface.

Significance to Biomedical Research and the Program of the Institute: The proposed research is significant in the field of gerontological research in that it is designed to specifically define the memory cell deficiencies of the aged mouse. Such a definition would be required before any attempt at antigen-specific immunological reconstitution of the aged animal could be undertaken.

Proposed Course: In vitro experiments utilizing the T and B-cell co-cultivation systems being developed will be used to assess the relative deficiencies (both immunological activity and turn-over) in the B and T-memory cell populations of the aged mice.

Honors and Awards:

Drs. Barbara E. Loughman, John J. Farrar and Albert A. Nordin presented a seminar related to this project to the Johns Hopkins Immunology Group on January 30, 1973.

Dr. Makinodan was invited to present a paper entitled "Cellular Nature of Immunodeficiency in the Aging" at the 2nd International Workshop on Primary Immunodeficiencies in Man held in St. Petersburg Beach, Florida, February 4-8, 1973.

Dr. Makinodan was invited to present a seminar at the Wistar Institute, Philadelphia, Pennsylvania on March 14, 1973.

Dr. Makinodan was invited to present a seminar at the Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana on May 16, 1973.

Dr. Makinodan was invited to present a seminar at the Department of Microbiology and Immunology, Temple University, Philadelphia, Pennsylvania on May 23, 1973.

Publications: None

Serial No.: HD-CCP-17

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Immunology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Pathology of Age-associated Kidney Disease of Wistar Rats Reared at GRC

Previous Serial Number: None

Principal Investigators: Katsuiku Hirokawa
Kenneth A. Rosenberg

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.7
Professional:	0.7
Other:	1.0

Project Description:

Objectives: The objective of these studies is to establish the nature of the kidney disease of the rats reared at GRC. The severity of the disease was studied as a function of the age of the rats and to determine if the immune activity is associated with the kidney lesions. Prevention or amelioration of the disease is of major interest in this project.

Methods Employed: Standard techniques suitable for light and electron microscopic observation were used.

Major Findings:

(1) Approximately 90% of the male and 50% of the female rats died of or with kidney disease. As expected, the severity and frequency of kidney disease is always greatest in the male rats of any age group.

(2) Kidney disease was detectable in some rats as young as three months of age whereas nearly every rat six months of age showed kidney disease manifested as focal glomerulitis.

(3) A slight deposition of Ig-G was observed along the glomerular basement membrane.

(4) Electron microscopic observation showed: (a) thickening of the glomerular basement membrane correlated with age, (b) rare occurrence of lumpy lesions, (c) cytoplasmic fusion of podocytes of glomeruli and (d) occasional abrasion of podocytes.

Significance to Biomedical Research and the Program of the Institute: Some diseases are thought to occur in aged animals, mostly or partly due to deficiency of immunological control. The kidney disease of rats reared at the GRC increases in its severity and frequency with age, and appears to have close relation with the age-associated decline of immune activity. An understanding of relationship between the decline of immune activity and increased occurrence of the disease in aged animals may enable us to prevent or ameliorate the disease.

Proposed Course: (1) Elution and characterization of kidney-fixed immunoglobulin. (2) Detection of factor(s) causing glomerulonephritis in the serum of rats. (3) Induction of experimental autoimmune kidney disease in both young and old rats by passive immunization with anti-rat glomerular basement membrane. (4) Studies on the permeability of the thickened glomerular basement membrane as compared to normal by using horseradish peroxidase, ferritin and immune complexes.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP-18
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Office of the Chief
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of Age on Steroid Hormone Binding and Responsiveness in Target Cells and Tissues

Previous Serial Number: None

Principal Investigator: George S. Roth

Other Investigator: Gary R. Dunn

Cooperating Units: None

Man Years:
Total: 1.2
Professional: 0.95
Others: 0.25

Project Description:

Objectives: A general characteristic of aging organisms seems to be an altered ability to respond to hormonal stimuli. Evidence from several laboratories suggests that a long overlooked possible explanation may lie in age-related changes in hormone uptake and/or binding by target cells and tissues. Efforts are currently underway in an attempt to determine (1) whether specific binding of steroid hormones by target cells and tissues is altered as a function of post-developmental age, and (2) whether such possible alterations can be correlated with age-associated changes in cell and tissue biochemical responsiveness to these hormones.

Methods Employed: A number of *in vivo* and *in vitro* assays will be employed to assess steroid binding, enzyme inducibility, viability, and various metabolic parameters in purified cell populations and tissues.

Major Findings: Muscle, brain and adipose tissue cytosols of adrenalectomized, male Sprague-Dawley rats exhibit progressively decreased glucocorticoid hormone binding *in vitro* from 2 to at least 24 months. Such reductions appear to be due to decreases in the number of specific hormone binding sites per unit mass (tissue weight and protein). Prostate glands yield similar reduced binding between 12 and 24 months. In contrast to other tissues, liver actually increases in binding sites at least until 12 months of age. Changes in numbers of glucocorticoid binding sites in liver and muscle have been related to changes in the inducibility of

tyrosine amino transferase by cortisol in vivo. Age-related changes in steroid binding do not appear to be due to the effects of small organic molecules since cytosols preadsorbed with activated charcoal yield binding results similar to those of untreated cytosols. Thus, binding alterations would seem to reflect quantitative and/or qualitative changes in cytosol macromolecules.

Brain cytosols of female mice aged 3 to 33 months exhibit progressively decreased numbers of estradiol and progesterone binding sites. Liver cytosols increase in these sites between 3 and about 18 months then decrease until at least 33 months. Such data are probably comparable to those obtained for adrenal glucocorticoids in rats since both sex steroids and corticosteroids have been reported to bind to the same sites in non-sex tissues. Uterine cytosol data are still uncertain since fertility cycle phases influence the number of binding sites, while ovariectomy has been reported to reduce this parameter.

Significance to Biomedical Research and the Program of the Institute: Binding of hormones to cell and tissue receptor sites is the initial step required for their physiological action. Present studies at this level offer an opportunity to elucidate the nature of the cellular and/or molecular phenomena which result in altered responsiveness in aged target tissues. Such information is essential to any attempts to alleviate or reverse age-related changes in biochemical vitality.

Proposed Course: Studies will attempt to further correlate age-related changes in hormone responsiveness with alterations in hormone binding by target cells and tissues. Emphasis will be placed on in vitro cell culture systems that will support growth of defined homogeneous cell populations. This approach should allow assessment of age-related changes in these functions at the level of tissue parenchymal and stromal cells. The ultimate goal of this investigation will be the determination of the mechanism(s) of such age-related sensitivity changes in hormone target cells.

Honors and Awards: None

Publications: Roth, G.S., and Adelman, R.C.: Age-dependent changes in sensitivity of rat submandibular gland in vivo prior to the initiation of isoproterenol-stimulated DNA synthesis. J. Gerontol. In press.

Serial No.: HD-CCP-19
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Mammalian Genetics Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effect of Chromosomal Aneusomy on Cellular Growth, Metabolism and Aging

Previous Serial Number: None

Principal Investigator: Edward L. Schneider (E.O.D. 5/23/73)

Other Investigators: Stuart Shorr
Gary Dunn

Cooperating Units: Roger Chakley, University of Iowa, Iowa City, Iowa
R. Yanoshevsky, Livermore Radiation Laboratory, Livermore, California

Man Years:

Total:	1.75
Professional:	1.25
Others:	0.5

Project Description:

Objectives: Experimental evidence indicates that there is a direct relationship between chromosomal aneusomy and aging in humans. Moreover, patients with aneusomies are characterized by retarded growth, decreased life spans and altered immune responsiveness. At the cellular level the fibroblasts and lymphocytes are deficient in their proliferative capacity and immunocompetence. The purpose of this project is to identify and elucidate cellularly and biochemically those alterations which impair the proliferative and immunological capacity of fibroblasts and lymphocytes in vitro. Another important goal of this project will be to investigate the relationship between advanced maternal age and chromosomal aneusomy.

Methods Employed: Fibroblasts and lymphocyte stocks will be prepared from patients with trisomy-21, 13 and 18 and normal controls. Cellular replications will be studied by measuring DNA synthesis, cell cycle time, and percent of replicating cells, and by examining cell cycle blockage, histone acetylation and phosphorylation, and DNA methylation. Ribosomal, messenger, transfer and mitochondrial RNA synthesis, turnover and degradation will be studied by utilizing polyacrylamide gel electrophoresis, pulse labeling and chromatographic methods. Protein synthesis will be examined by measuring incorporation of appropriate labeled precursors. Chromosomal

analysis will be performed of preimplantation and postimplantation mouse embryos from young and old mothers.

Major Findings: This project was initiated late in FY 1973 and therefore much of the effort was devoted to the creation of the laboratory and animal farm facilities and standardization of the various experimental procedures.

Significance to Biomedical Research and Program of the Institute: Information derived from this program should have a tremendous impact on cellular biology of aging, for the proliferative capacity of certain somatic cells is one of the major functional properties that seem to deteriorate with age. In addition, these studies will provide insight into the deranged cellular metabolism in trisomy-21. Lastly, this work will provide an animal model for studying the chromosomal aneusomies which are maternally age related in man (Down's syndrome, Turner's syndrome, Klinefelter's syndrome, XXX, and XYY).

Proposed Course: Initially emphasis will be placed on cellular and biochemical characterization of the impairment in proliferative capacity observed in human trisomy-21 fibroblasts and lymphocytes. Subsequently other naturally occurring trisomic and aneusomic cells will be analyzed in conjunction with experimentally induced aneusomic cells.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP-20

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Mammalian Genetics Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effects of the Agouti Locus on Development and Aging in Mice

Previous Serial Number: None

Principal Investigator: Gary Dunn

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.60
Professional:	0.50
Other:	0.10

Project Description:

Objectives: An understanding of the genetic regulatory mechanisms operating in cells is of basic importance for our understanding of age-related decline in cell function, especially of genes which are expressed throughout the life span of an organism. Our model system is the agouti locus of the mouse and specifically the yellow-agouti allele, A^y. Homozygotes for this particular allele die as early embryos while heterozygotes lack black pigment in their hair, are obese, and have a shortened life span relative to their normally pigmented littermates. To date, our research has centered on obtaining a fuller understanding of the homozygous lethality of A^y.

Methods Employed: The technique of experimental delay of the implantation process in gestation has been used. This involves ovariectomy once the female has become pregnant and maintenance of the preimplantation status of the uterus by progesterone injection. Implantation can then be induced at will by the administration of estrogen.

Major Findings: These studies appear to indicate that A^y/A^y embryos die during a fixed time period after fertilization irrespective of whether or not the process of implantation is occurring. This suggests that homozygosity for A^y may be inhibiting some function of all cells in the embryo rather than just those cells (trophoblast) responsible for implanting the embryo in the uterine epithelium as previously thought. The suggestion that A^y inhibits some function in all cells when homozygous is also

consistent with its observed heterozygous effects which were not explained by the theory that it acts only on trophoblast.

Significance to Biomedical Research and the Program of the Institute: The agouti locus appears to control some basic cellular process which is required for normal development and proper physiological functioning throughout life. The agouti gene product appears to be involved in regulatory mechanisms which affect many or all organ systems. The shortened life span of yellow-agouti mice suggests that abnormalities in such a system may be a cause of the process of aging and that such abnormalities can be genetically determined.

Proposed Course: Studies on the embryological effects of yellow-agouti will be continued since it is possible to examine the homozygous effects of this locus only during early development. However, the manifestations of this mutation in adult heterozygotes will also be studied. The adrenal glands of these animals are known to hypertrophy with age and this process is correlated with the development of obesity. Therefore, experiments will be done to determine whether the mechanism for this hypertrophy is due to a defect in the adrenal glands or in the hypothalamic-pituitary control of adrenal function. Since hypertrophy suggests elevated hormone production, these experiments will involve measurement of adrenal hormone levels in ACTH stimulated and unstimulated animals. Such experiments will better define the nature of the endocrine defect in yellow-agouti mice. Diet has also been shown to affect the development of obesity and the life span of yellow-agouti mice. The possibility that these effects are mediated by the adrenal gland will be examined by assaying adrenal hormone levels in the blood under different dietary conditions.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP2

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Environments and Genetics Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Dietary Proteins and Longevity

Previous Serial Number: Same

Principal Investigator: Charles H. Barrows, Jr.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.50
Professional:	.75
Other:	.75

Project Description:

Objectives: Restricted dietary protein intakes may increase life span by (1) retarding differentiation and growth and (2) delaying the onset of diseases associated with late life. Therefore, in this series of experiments, attempts were made to determine (1) the extent to which an inhibitor of protein synthesis, viz., cycloheximide, would retard the development of chick embryos; (2) whether longevity can be increased by decreasing dietary protein in adult organisms, i.e., 16 month old rats and (3) whether a reduced dietary protein intake would change the incidence and/or time of onset of leukemia in AKR mice.

Methods Employed: (1) Attempts to regulate differentiation and growth in young growing animals were carried out by injecting 0.8% or 1.0% of cycloheximide into one day old chick embryos. The levels employed here did not result in an increase in mortality or frequency of anomalies of the animals. When the embryos were 2, 3, and 4 days old, the variables measured were size, heart rate, and the degree of differentiation as described by staging according to Hamburger and Hamilton. In older organisms (viz., 8, 11, 14, and 17 day old embryos), DNA, protein, as well as various enzymes (acetyl cholinesterase, cytochrome C oxidase, alkaline and acid phosphatase, and cathepsin) were determined by standard procedures. (2) In order to determine whether longevity could be increased by decreasing dietary protein in adult organisms, 16 month old rats were offered diets which contained either 24, 12, 8 or 4% protein. The mortality statistics

on these animals were determined. (3) AKR mice were fed diets which contained 26, 4, 3 or 2% protein and efforts made to establish, by gross pathology and histological examination, whether the increase in life span associated with dietary protein restriction was associated with a change in the incidence and/or the onset of leukemia in these animals.

Major Findings: (1) Injection of cycloheximide resulted in a reduction in the rates of differentiation and growth at 2, 3, and 4 days. In the older organisms a reduction in body weight was observed. However, the activities of alkaline phosphatase, acid phosphatase, cathepsin, cytochrome C oxidase and acetyl cholinesterase expressed either as per unit wet weight, protein, or DNA was not affected. (2) Reduction in the dietary proteins at 16 months of age from 24 to 8 or 4% did not result in an increase in longevity. However, a reduction in dietary protein to 12% resulted in a 24% increase in life span, (27.6 ± 2.0 weeks to 34.2 ± 1.7 weeks) ($P = 0.01$). (3) Mortality data in AKR mice indicate 50%, 20%, 8% and 40% in the animals fed 26%, 4%, 3% and 2% dietary protein respectively. Thus far, on the basis of gross pathology, all mortality was associated with leukemia with the exception of the animals fed the 2% protein diet in which case malnutrition appears to be the cause of death.

Significance to Biomedical Research and the Program of the Institute: These dietary manipulations may result in the retardation of the onset of various diseases and biological aging.

Proposed Course: The dietary protein intake will be reduced in 16 and 3 month old rats from 24 to 12, 8 or 4% and differences in life span will be established. The incidence of kidney pathology and age-associated changes in renal function will be assessed.

Honors and Awards: None

Publications: Barrows, C.H., Jr.: Nutrition, aging, and genetic program. Amer. J. Clin. Nutrit. 25: 829-833, 1972.

Serial No.: HD-CCP-21

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Environments and Genetics Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Effect of Dietary Manipulations on Life Span, Incidence of Diseases and Immunologic and Related Functions

Previous Serial Number: None

Principal Investigators: C. H. Barrows, Jr.
K. Hirokawa
T. Makinodan

Other Investigators: G. S. Roth
A. A. Nordin
J. J. Farrar
M. L. Heidrick
G. R. Dunn

Cooperating Units: None

Man Years:

Total:	1.20
Professional:	.95
Other:	.25

Project Description:

Objectives: Levels of intakes of various nutrients ingested at specific intervals of the life cycle which will optimize physiological performance are unknown. Perinatal undernutrition results in irreversible changes which markedly affect the organism throughout its life. Dietary restriction imposed immediately following weaning results in an increased life span of rodents with average life expectancies. However, no information is available if this latter effect can be brought about in rodents when the dietary restriction is imposed during adulthood or in animals with long life expectancies. More important is that the cellular mechanisms which regulate these observations are not known. Since the immune system plays a major role in the maintenance of health throughout the total life span and is well defined at both the cellular and molecular levels, the effect of various dietary manipulations imposed at specific ages will be assessed primarily on the immune system of mice with average and long life spans.

Methods Employed: Various strains of mice will be fed, ad libitum, diets of nutritionally defined composition but varying in their protein content during either pregnancy, lactation, growth or adulthood. Actuarial and pathological studies will be carried out. These data will be correlated with assessments of the regulation of both cell mediated and humoral immune activities. In addition, the turnover of total proteins and specific enzymes in liver and muscle will be estimated enzymatically and immunologically. Therefore these data will provide information regarding the interrelationship among the enzymatic activity, immunocompetence, turnover of a number of specific proteins and the life span of the organism. The effect of dietary manipulations will also be related to age-associated changes in the specific binding of hormones in cells and in the cellular responses by measurement of enzymatic induction and glucose uptake.

Significance to Biomedical Research and the Program of the Institute: Dietary manipulation could result in improvement of the immune activity and a decline in the incidence of immunodeficiency diseases of the aged.

Proposed Course: Initially the effect of dietary protein intake on the regulatory factors in the immune system and on other physiological functions will be assessed. Other dietary manipulations, including the effects of saturated and unsaturated fats, calories and heavy metals will follow.

Honors and Awards: None

Publications: None

Serial No.: HD-AG13

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Environments and Genetics Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Aging in the Rotifer

Previous Serial Number: Same

Principal Investigator: N. Meadow

Other Investigator: C. H. Barrows, Jr.

Cooperating Units: None

Project Description: This project has been discontinued.

Publications: None

Serial No.: HD-CCP-22

1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Morphology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ageing and Genetically Defective Mammalian Skeletogenic Cells and Connective Tissues

Previous Serial Number: None

Principal Investigators: Dorothy F. Travis

Other Investigators: None

Cooperating Units: Laboratory of Biochemistry
National Institute of Dental Research
Bethesda, Maryland

Johns Hopkins University
Baltimore, Maryland

Man Years:

Total:	0.30
Professional:	0.15
Other:	0.15

Project Description:

Objectives: The broad objectives of this program are directed at the underlying mechanisms associated with changes in mitotic and post-mitotic skeletogenic cells and connective tissues in genetic models and with age in mammalian tissues including human and experimental mammalian model systems.

Methods Employed: In these studies biochemical, ultrastructural, cytochemical, radioautographic and other procedures will be used to investigate these tissues in vivo and in vitro.

Significance to Biomedical Research and the Program of the Institute: The significance of these studies will rest on their contribution to the general conceptual understanding of the poorly delineated cellular and subcellular mechanisms which regulate the changes that occur in both non-mineralized and mineralized collagenous connective tissues of humans and other mammals with age and as a result of genetic defects.

Proposed Course: New project.

Honors and Awards: None

Publications: None

Serial No.: HD-CCP1
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Morphology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Changes in Cells and Extracellular Matrices of the Crayfish Gastrolith Disc during Growth, Development and Aging

Previous Serial Number: Same

Principal Investigator: Dorothy F. Travis

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	0.35
Professional:	0.10
Other:	0.25

Project Description:

Objectives: The purpose of this study has been to establish poorly understood structural and functional principles in mitotic and post-mitotic gastrolith epidermal skeletogenic cells during growth, development and ageing as these principles relate to: (a) synthesis, organization, and mineralization of the structural components of the matrix and (b) mineral metabolism including cellular storage and release mechanisms.

Methods Employed: Ultrastructural and electron cytochemical, both modified and non-modified, procedures have been used in mineralized and EDTA demineralized mitotic and post-mitotic skeletogenic tissues. Differential enzymatic digestion of the structural components of the gastrolith matrix (flamenous material and matrix vesicles) and tissue components (precursors and/or structural) to characterize and identify the chemical nature of these components with age are being carried out by the method of Bodely and Wood (1972).

Major Findings: In growing mitotic skeletogenic cells, results indicate that (1) the function of lysosomes differs in epidermal cells: in feeding animals they function in normal maintenance activities, turnover of cell components, while in pre-molt, non-feeding animals, they function in degradation of basal regions of the cell that contain stored glycogen, lipid, and large protein crystals and in turnover of resorbed products from the old exoskeleton for resynthesis of gastrolith matrix structural components; (2) synthesis of sterols, which constitute 4% and phospholipids

which constitute about 5% of the structural components of the gastrolith matrix, probably occur during pre-molt in the extensively and newly developed apical smooth ER; (3) the carbohydrate moiety of filament material in the gastrolith matrix is synthesized in the Golgi where mineral particles also are bound to this structural moiety and are transported by Golgi vesicles to the cell surface for release; (4) mitochondria in pre-molt cells concentrate, store, and transport complexed inorganic calcium to the cell surface for release in the developing gastrolith; (5) pre-molt cells release alternately filamentous material or matrix vesicles from the cell surface into the developing gastrolith. The exocytotic matrix vesicles, which arise from the plasma membrane and can account for the 5% phospholipid of the gastrolith matrix, contain amorphous mineral particles and very small crystallites and appear to serve as primary nucleating sites for amorphous calcium carbonate and calcite in the gastrolith matrix. These gastrolith matrix vesicles are distinctly analogous structurally and functionally to those that arise from mammalian chondrogenic, osteogenic, and dentogenic cells, which also function as primary nucleating sites of hydroxyapatie in these tissues. Aged post-mitotic gastrolith epidermal cells differ markedly from young dividing cells in their extensive accumulation of lipid and degradation of mitochondria, swelling of the RER, and virtual disappearance of the cytoplasmic matrix; their adjacent basement membrane has thickened five-fold, mimicing reported age-related changes in the mammalian kidney. Furthermore, the aged animal shows marked changes in organization and infiltration of the structural components of the non-mineralized extracellular matrix with lipid and marked degradation and loss of cells in the sub-epidermal connective tissue. Several manuscripts relating to these studies are being prepared for publication.

Significance to Biomedical Research and the Program of the Institute: Both non-mineralized and mineralized connective tissues of mammals and the few invertebrate model systems studied show changes in organization, amount, composition, degree of cross linking, flexibility, permeability and rigidity of the structural components with age, which severely affects the functional capacity of the aged animal. To pinpoint the causes for these changes demands an understanding of poorly delineated cellular and subcellular mechanisms which regulate these changes. The establishment of cellular mechanisms in mitotic skeletogenic cells which synthesize and mineralize the extracellular connective tissues has clearly placed the primary role of regulation of mineral metabolism including concentration, storage, transport and release of the inorganic mineral phase on the call directly and not the extracellular matrix per se. These established principles make possible the marked contrast with post-mitotic, functionally impaired aged cells.

Proposed Course: Further studies on this are being directed at understanding the cellular mechanisms that underly the marked age-related changes in these post-mitotic skeletogenic cells and connective tissues. This project is being phased out.

Honors and Awards: Dr. Travis serves as a member of the Council of the Washington Society of Electron Microscopy.

Dr. Travis serves as an Advisory Editor to the journal,

Calcified Tissue Research.

Publications: None

Serial No.: 4D-CCP11
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Morphology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Ultrastructural Studies of the Regenerating Sea Spine During Growth and Developmental Sequences

Previous Serial Number: Same

Principal Investigators: Barry M. Heatfield
Dorothy F. Travis

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.05
Professional:	1.05
Other:	0.0

Project Description:

Objectives: The purpose of this investigation was to delineate cell types in young regenerating spine tissues with the specific aim of identifying the skeletogenic cell type so that subsequent age-related changes could be studied in this cell of the non-growing spine stub.

Methods Employed: Modern cytological and cytochemical techniques and procedures of both light and electron microscopy are being utilized.

Major Findings: The results indicate that in addition to numerous epidermal and dermal cell types, including those resembling coelomocytes which contain large, intracellular spherules, there are young, active skeletogenic cells, the "calcoblasts." Calcoblasts were characterized by numerous pseudopodia and a conspicuous nuclear protrusion encircled by the Golgi complex. These cells extend a thin, cytoplasmic skeletal sheath, which intimately surrounded the growing surfaces of each of the calcite microspines comprising the young skeleton. Distally, the sheath enclosed an extracellular channel containing an amorphous matrix in which skeletogenesis occurs.

This intimate morphological relationship between the young calcoblast and the growing mineral surface indicates that calcoblasts directly control skeleton morphogenesis and regulate the kinetics of skeletal growth,

functioning in a manner similar to osteoblasts in vertebrate bone.

The identification, for the first time, makes possible subsequent studies of age-related changes in this skeletogenic cell in old, non-growing calcified spine stubs.

Significance to Biomedical Research and the Program of the Institute: The results of these studies have a direct relevance to our understanding of calcifying systems, particularly vertebrate bone, because the available evidence suggests that similar mechanisms are operative in a wide variety of calcified tissues. Moreover, the continuing study of concomitant changes in structure and function of the calcoblast cell-type throughout developmental sequences will contribute to our understanding of age-associated changes in calcified tissues generally.

Proposed Course: Age-associated, ultrastructural changes in calcoblasts are being investigated by comparing this active skeletogenic cell in the regenerating spine tip, with those in the old, non-growing stub. This project is being phased out.

Honors and Awards: None

Publications:

Heatfield, B.M., and Travis, D.F.: The fine structure of calcoblasts in the regenerating sea urchin spine. In Arceneaux, C. J. (Ed.): 30th Ann. Proc., Electron Microscopy Soc. America. The Proceedings, pp. 162-164, 1972.

Cavies, T.T., Crenshaw, M.A., and Heatfield, B.M.: The effect of temperature on the chemistry and structure of echinoid spine regeneration. J. Paleontol. 46: 874-883, 1972.

Serial No.: HD-CCP8
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Morphology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Age-related Changes in Mammalian Myocardial Cells

Previous Serial Number: Same

Principal Investigators: Dorothy F. Travis
Trexler M. Topping
Toshihide Sato

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 2.35
Professional: 1.75
Other: 0.6

Project Description:

Objectives: The broad objectives of this program have been directed at understanding basic structural and functional principles underlying the activities of myocardial cells during developmental sequences and aging process. Encompassed in these considerations of highly differentiated post-mitotic cells, studies have been carried out on both left ventricular mural and papillary myocardial cells.

Methods Employed: Ultrastructural and electron cytochemical, both modified and non-modified procedures, have been used in these studies of 6, 12, and 24 month old male rats.

Major Findings:
A. The left ventricular mural myocardial cells. The purpose of this investigation was to elucidate mechanisms of lysosomal activity in the rat left ventricular mural myocardium, mechanisms not understood in any mammalian myocardial cells. Results indicated: (1) Two types of primary lysosomes, showing differences in acid hydrolase activity. (2) Two different pathways of synthesis, transport, and packaging of primary lysosomes. (a) Nuclear pole zone. Synthesis occurs in cisternae of the RER or granular regions of the nuclear membrane or directly from other elements of the smooth ER to the Golgi cisternae for packaging and release from the swollen end sacs. (b) At distal cell sites from the Golgi, synthesis occurs in RER; transport, packaging, and release occurs directly from

smooth ER, bypassing the Golgi. (3) Two lysosomal mechanisms of autophagic mitochondrial degradation. (a) By matrix densification to form residual bodies. (b) By matrix clarification to form residual bodies. (4) Lipid droplets were degraded by lysosomal and auto-oxidative activity to form residual bodies, all of which show acid hydrolase activity. This is the first investigation to establish these mechanisms in any mammalian myocardial cells. The establishment of these mechanisms bears directly on age-related changes in cellular structure and function. The latter encompasses lysosomal synthesis and turnover rates, membrane phenomena in selection of components for breakdown, causal factors of residual body accumulation, and their different enzyme content with age.

B. The left ventricular papillary myocardial cells.
Age-related changes in mechanisms of lysosomal degradation in the rat left ventricular papillary myocardial cells. The purpose of this investigation was to elucidate mechanisms responsible for the marked age-related changes evident in 24 month old rat papillary myocardial cells in regions of the muscles in which the cells are grossly normal in structural appearance but show marked changes over the 6 and 12 month old cells. Changes evident in old cells were in: (1) the structural configuration of the nucleus; (2) mitochondria, with hypoxic or anoxic swelling, clarification of matrix, separation and disorganization of the cristae in some 12 and in most 24 month old cells; (3) lysosome and residual body accumulation; (4) mechanisms of autophagic degradation of mitochondria by redundant smooth ER segregating membranes; (5) lipid droplets with increased electron density and often degraded in autophagic vacuoles by lysosomal enzymes and perioxidation. These results indicate that slow subtle changes occur in the ageing myocardial cell. Consequences of slow but evident hypoxic or anoxic changes in mitochondria could result in curtailed oxidative metabolic capacity, ion leakage and failure to membrane transport systems, inability of myofilaments to function normally, greater production of lysosomes with greater fragility or altered permeability of membranes, permitting slow leakage of acid hydrolases into the cytoplasmic matrix. Lipid droplet degradation in the old cells by perioxidation might similarly cause lysosomal and other membrane damage by leakage of free peroxide radicles. Nuclear changes may also reflect acid hydrolase leakage leading to altered DNA and RNA at the genome or at the transcription levels and synthesis of defective proteins. Changes in the nature of lipid droplets with age suggests that altered membranes of the smooth ER could synthesize faulty lipids. Faulty macromolecules produced at these sites might well be responsible for the production of seemingly redundant segregating membranes in the old cell. We feel that it is these slow but subtle changes, and not residual body accumulation per se, with time that alters the cell's capacity to function normally. This manuscript is prepared for review and will be submitted to the Journal of Ultrastructure Research for publication.

Rat left ventricular papillary myocardial cell death. The purpose of this investigation was to elucidate the mechanisms responsible for myocardial cell death in regions of the muscles in which the old 24 month cells are grossly abnormal structurally, showing necrosis and fibrotic replacement. Results indicate that: (1) further, marked changes in the nucleus were evident; (2) most mitochondria showed extreme hypoxic or anoxic changes with reduction or loss of cytochrome oxidase activity; (3) increases in primary but decreases in secondary lysosomes and residual bodies were

evident; (4) acid hydrolase activity was evident in the cytoplasmic matrix indicating leakage and loss from secondary lysosomes and residual bodies, thus accounting for their decreases in these severely affected cells; (5) myofilaments were degraded and Z-band thickening, disorganization and disruption were evident; (6) swelling of the T-tubules, cytoplasmic vacuolation and breakage of the plasma membrane were evident; (7) Macrophages were trapped in the process of consuming both normal and abnormal regions of the same cell; (8) invading fibroblast and collagen fiber replacement of dead cells were evident. These results clearly indicate that slow, subtle changes have occurred in the ageing papillary myocardial cell and that consequences of such changes as suggested previously have in fact occurred. Physiological mechanisms that start the chain of events may well be: (1) ventricular hypertrophy causing mechanical stress and strain on the papillary muscles and (2) reduced coronary blood flow with age, the papillary muscles being the last to be supplied. This latter factor alone could cause slow hypoxia or anoxia, setting off the entire chain of events. The extensive cell death and fibrotic replacement with age in the papillary muscles would drastically alter the functional capacity of the heart. This manuscript is being prepared and will be submitted to the Journal of Ultrastructure Research for publication.

Significance to Biomedical Research and the Program of the Institute: More than one-half of all human deaths in the U. S. occur secondary to cardiac disease. This demands an understanding of cellular mechanisms responsible for age-related decrements in valvular and cardiac function. The establishment of mechanisms of lysosomal activity in the rat left ventricular mural myocardium are the first to be established in any mammalian myocardial cells. Similarly, the elucidation for the first time of cellular mechanisms responsible for the marked age-related changes that occur in old papillary myocardial cells and those responsible for papillary myocardial cell death have significant relevance to the understanding of decrements in valvular and cardiac function of older humans and other mammals.

Proposed Course: Further studies require the biochemical quantitation of age related lysosomal and mitochondrial enzymatic activities in sub-cellular fractions of the left ventricular papillary myocardial cells.

Honors and Awards: Dr. Travis was invited to speak in a seminar at Georgetown University, School of Medicine, Department of Anatomy, Washington, D. C.

Publications: Topping, T.M., and Travis, D.F.: An electron cytochemical study of mechanisms of lysosomal activity in the rat left ventricular mural myocardium. J. Ultrastruc. Res. In press.

Serial No.: HD-AG37
1. Gerontology Research Center
2. Laboratory of Cellular and Comparative Physiology
3. Morphology Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Studies on the Comparative Physiology of Ageing and Environmental Effects on the Ageing Process

Previous Serial Number: Same

Principal Investigator: Mary Anne Brock

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: (1) To delineate both endogenous rhythmic changes and temperature-induced changes in the longevity of poikilothermic and homoiothermic animals; (2) To define the age-related structural and functional characteristics of pre-and post-mitotic cell types as these relate to cellular mechanisms associated with changes in the rate of ageing.

Methods Employed: Colonies of the marine coelenterate Campanularia flexuosa were cultured in aerated, artificial sea water at 4°, 10°, 17° and 24° C. for ageing studies and other determinations at each of these temperatures. Golden hamsters, Mesocricetus auratus, were maintained either in a 24° C. environment or periodically exposed to 5° C. to permit hibernation. Ultrastructural and biochemical techniques were used.

Major Findings:

A. Endogenous rhythms in the marine invertebrate, Campanularia flexuosa. The delineation of endogenous rhythms in C. flexuosa longevity challenges the generally accepted concept of specific age, an age at death which is characteristic of the species. It generally has been assumed that vertebrates and invertebrates grown under constant environmental conditions have an invariable life span. However, a contradiction to this concept is raised by the existence of rhythmic changes in C. flexuosa hydranth longevity.

1. Annual (cirannual) rhythms in Campanularia. Endogenous circannual rhythms in growth, development and longevity of the marine coelenterate Campanularia flexuosa have persisted for over three years in the absence of periodic signals from the environment. The free-running periods for the cycles at three constant ambient temperatures, 10°, 17° and 24° C., approximated one year, but were always more than 365 days. Temperature switches at different times of the annual cycle have shown differential seasonal sensitivities to temperature in both hydranth development and longevity; these and related data have implicated temperature as a Zeitgeber.

The endogenous circannual rhythms were marked by seasons of luxuriant colony growth that alternated with lulls in growth and development. During luxuriant growth, development of individual hydranths was initiated regularly in new positions and as replacements at regression sites on uprights. Nearly all hydranth development was normal and adult life spans were long. The profuse growth was interrupted by seasons of repressed growth each summer and punctuated by recurrent, short periods of sharply curtailed growth in mid-winter. During repressed growth, the rate of hydranth initiation at all sites was depressed, and abnormal development aborted many individuals. Most strikingly, hydranth life spans fell to about half those observed during seasons of prolific growth. With the spontaneous return to the luxuriant growth habit, hydranth life spans rose, nearly doubling.

To further define the endogenous control of the circannual cycle, new colonies of C. flexuosa were obtained from their natural environment in late 1971 and cultured together with the older colonies in the same vessels. Despite this intimate association, the endogenous rhythms of the new colonies were clearly out of phase with the same rhythms observed in the long-term laboratory-maintained colonies, for they exhibited expansive growth when the growth of the older colonies was sharply curtailed.

2. Lunar rhythms in Campanularia. Hydranths of Campanularia not only exhibit rhythmic and predictable annual phases of increased longevity but also persistent lunar rhythms in hydranth development and longevity. Within a lunar cycle, three-to four-fold differences in life expectancy were observed. Each cycle began with the development of long-lived hydranths, and progressively shorter-lived individuals developed each day as the cycle unfolded. The cycles overlapped and have continuously repeated for over three years. Analysis of the lunar cycles as well as their relationship to the circannual rhythms is being completed.

B. The effect of hibernation on the longevity of golden hamsters. Preliminary studies have shown a marked effect on the longevity of animals which have hibernated, their body temperature falling to near 6° C. during the hibernating season. These studies suggest that there is a marked extension of the life span associated with hibernation and that hibernation may have a more profound effect in increasing the longevity of male hamsters. Furthermore, the extension of the life span appears to bear no simple direct relationship to the length of time in hibernation. This has been clearly demonstrated with the use of a remote monitoring system that

was developed for these studies and has successfully recorded the precise length of time each hamster spent in hibernation. The system depends on individual infra-red sensors positioned over each animal with leads to a multichannel scanner and recorder.

Significance to Biomedical Research and the Program of the Institute: A primary objective of gerontologists is to extend the number of continuous years of productive, optimally functional and enjoyable life. To achieve this goal, an understanding of cellular functions in regulatory mechanisms associated with increased longevity such as that observed in vertebrates (mammals) and invertebrates either at reduced temperatures or during certain phases of endogenous rhythms in longevity is essential. The delineation of these regulatory mechanisms has significance in their possible applicability in manipulating the rate of cellular ageing in other mammals, including man. Furthermore, this first delineation of endogenous rhythms in longevity is of significance not only to the field of ageing but in relation to similar yearly or seasonal rhythms that alter physiological and biochemical parameters such as ventilatory capacity and the immune response in mammals, including man. Circannual rhythms may well be as universal as circadian rhythms and of profound importance in biomecial research.

Proposed Course: A series of papers on annual and lunar rhythms in C. flexuosa longevity are in preparation. This project is being phased out. Studies on age-related considerations of mammalian hemopoietic connective tissue will be initiated.

Honors and Awards: Dr. Brock was elected to the Board of Governors of the Society for Cryobiology.

Dr. Brock served as Secretary of the Society for Cryobiology.

Dr. Brock was an invited speaker at the 1972 AAAS Symposium, Developmental Biology of the Cnidaria.

Dr. Brock was invited to speak at a AAAS Symposium on Annual (Circannual) Biological Clocks.

Publications: Brock, M. A.: Growth, developmental and longevity rhythms in Campanularia flexuosa. Amer. Zool. In press.

NICHD ANNUAL REPORT
July 1, 1972 through June 30, 1973
Gerontology Research Center
Laboratory of Molecular Aging

The Laboratory of Molecular Aging has developed scientific data and theory fundamental to the understanding of molecular processes involved in aging. Research projects, utilizing a variety of model systems, have examined questions related to regulatory processes in the transmission of genetic information, mechanisms of control of enzymatic activity and cellular metabolism, and biochemical changes during aging that alter the responses and functions of subcellular organelles and cells.

An extensive study of infection of human cells (WI-38) in culture by vesicular stomatitis virus and its prevention indicates: (1) Senescence of host cells is not detrimental to viral replication with the exception of a brief period before cellular death. (2) Cellular viral defense, i.e., the induction of interferon, is functional throughout the life span of the cells up to the moribund state, but its efficiency, i.e., the amount of poly IC required for induction, decreases with age. (3) In senescent cells the polynucleotide inducer of interferon produces the antiviral state faster than treatment with interferon itself, suggesting either another antiviral protection mechanism in senescent cells or a change in the relative rates of uptake of poly IC and interferon in the senescent cell. Related studies with this experimental model show that antiviral activity of the complex of poly IC is more critically influenced by substitutions in the inosinate than in the cytidylate strand. Significantly, it is found that poly IC anchored to solid carriers suspended in the medium over the cell monolayer conveys antiviral protection.

Synthetic analogs of nucleic acids, representing modifications of the structure of native genetic material, have been shown to interfere with specific biological processes and, therefore, can be used as probes to study biochemical mechanisms. Thus, polyvinyl uracil and polyvinyl adenine both inhibit murine leukemia virus infection, but they are rather ineffective against Sinbis and vesicular stomatitis viruses. The inhibition of the leukemia virus appears to be related to the inhibition of a RNA-dependent DNA polymerase. Polyvinyl uracil also inhibits protein synthesis in vitro and it is toxic to rapidly growing cells. On the other hand, polyvinyl adenine does not inhibit cell multiplication nor host cell synthesis of nucleic acids and proteins.

Complexes of platinum have been used to probe the mechanism by which the replication of DNA and synthesis of RNA are frustrated by metal ions. It is known that the cis-form of a platinum complex inhibits DNA replication, cell division, and is an effective antitumor agent, whereas the trans-form of the complex is not active in these respects. It has now been found that the cis-isomer, but not the trans-isomer, forms complexes with DNA. The Pt-containing complex inhibits transcription in an in vitro system, and this inhibition is on the polymerization process, without affecting the initiation process. A technique has been developed to replace the zinc, the natural metal in RNA polymerase, with several metals including copper and to retain enzymatic activity. The substitution of copper, allowing electron spin resonance studies, may provide a valuable tool in elucidating the molecular action of the enzyme.

The interactions of synthetic polypeptides with DNA have been used to examine the nature of histone binding in chromatin. Physicochemical studies indicate that polylysine binding to DNA resembles that of histone I, and the similarity is augmented by introducing alanine residues into the polylysine (alanine-lysine copolymer). Polyarginine, on the other hand, resembles histone IV in its binding to DNA. Basic polypeptides with long side chains form weaker links to DNA than shorter chain polypeptides. DNA that is not bound to polypeptide components appears to be required for the regulation of the conformation of polypeptide-bound DNA. This observation could explain the fact that only part of the DNA in chromatin is covered by protein, while all of the chromatin is repressed toward in vitro transcription.

Studies on the mechanism of active transport of sugar in the kidney have continued to make significant progress and several underlying principles have been established. Glucose transport by isolated renal brush border membranes is comprised of at least two processes, a high affinity binding system and a carrier-mediated transport system. The presence of a stereospecific high affinity binding system has been unequivocally demonstrated by equilibrium dialysis. Kinetic studies of the binding have characterized its time course, reversibility, saturability, susceptibility to the presence of phlorizin, and have determined the effects of Na^+ as well as Ca^{2+} and Mg^{2+}. A particulate fraction derived from detergent-extracted brush border membranes has been prepared. This preparation has the same affinity and capacity as intact membranes but has lost about 90% of its protein content and shows no evidence of vesicular structure. Evidence for a glucose-bound protein has been obtained. The mechanism of uptake of high concentrations of glucose by the intact brush border membranes shows both influx and efflux components and is consistent with a hypothesis for a carrier-mediated transport into and out of vesicles. Prior loading experiments demonstrate the phenomenon of counter-transport or facilitated exchange diffusion. Although Na^+, per se, is not required for uptake of glucose by the brush borders, the rate of glucose transport by the membrane is greatly enhanced by a Na^+ gradient from the outside to the inside of the membrane.

Extensive studies on the control of pyruvate oxidation in mitochondria from blowfly flight muscle, and confirmed with mitochondria from rabbit heart muscle, have led to a novel mechanism for the regulation of Krebs cycle activity. Both ADP and inorganic phosphate are required for the uncoupled (FCCP) oxidation of pyruvate as well as for the coupled oxidation. The FCCP-stimulated oxidation is markedly inhibited by physiological levels of Ca^{2+}. The apparent K_i = 10 μM. The electron transport chain of the mitochondria is fully functional in the presence of Ca^{2+} since the oxidation of α-glycerolphosphate is not inhibited, indicating control at the dehydrogenase level. Kinetic studies show that the Ca^{2+} inhibition is uncompetitive with respect to ADP and FCCP, but complex, and noncompetitive with respect to pyruvate. The results of these and other studies suggest that pyruvate oxidation and Krebs cycle activity is limited by NAD-dependent isocitrate dehydrogenase activity and Ca^{2+} plays a role in its regulation. An additional mechanism for the control of pyruvate metabolism has been demonstrated in mitochondria for the first time. In analogy to glycogen phosphorylase, this involves the phosphorylation and dephosphorylation of the pyruvate dehydrogenase complex by a kinase and phosphatase, respectively. In contrast, however, with pyruvate dehydrogenase the phosphorylated form is inactive whereas the dephosphorylated form is active.

A correspondence between the decline in the ability of aged blowflies to fly and in the maximally stimulated respiratory rate (State 3) and respiratory control ratio of flight muscle mitochondria was reported previously. The biochemical mechanism and locus of this decrement with senescence has been investigated further by examining the bioenergetic properties of mitochondria from mature and aged flies for rates of oxidation during coupled oxidative phosphorylation, activities of the dehydrogenases, concentrations of components of the electron transport chain, phosphorylation of ADP, and the coupling mechanism, itself. Evidence, to date, indicates that the specific activities of α-glycerolphosphate and pyruvate dehydrogenases are unaltered by age. Similarly, the concentrations of cytochromes a, a_3, b, and $c+c_1$ are the same for both age groups. ATP synthetase, measured as ATPase, is unchanged with senescence. In contrast, the uncoupled oxidation of substrates is significantly decreased with aging, showing the 30-40% decrement between mature (7-10 days) and aged (31-33 days) as seen previously for the maximally stimulated coupled rate of respiration. These observations narrow the possible loci of the defect in aging to the bioenergetic system between the primary dehydrogenase and the FCCP-sensitive uncoupling reaction.

Hormonally (parathyroid, synthetic 1-34, and catacholamine) stimulated adenyl cyclase has been demonstrated in renal cortical membranes. Luminal brush border membranes bind c-AMP, as determined by equilibrium dialysis and Millipore filtration experiments. Kinetic parameters of the binding have been examined. It is anticipated that the binding of cAMP may initiate kinase activation and potentially exert a controlling role in renal tubular function.

Serial No. HD-GMA1
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Interaction of Metal Ions with Polynucleotides and Related Compounds

Previous Serial Number: Same

Principal Investigator: G. Eichhorn (35% time)
J. Rifkind (40% time)
Y. Shin (40% time)
R. Srivastava (100% time)
E. Tarien (70% time)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	3.75
Professional:	2.85
Others:	.90

Project Description:

Objectives: (1) To understand the biological activity of metal complexes of nucleotides and polynucleotides on the basis of their structures and reactions. (2) To find specific reactions of the individual nucleotides. (3) To understand the biological significance of metal ions in nucleic acid function.

Methods Employed: The interaction of metal ions with polynucleotides and their monomeric components is studied, using a variety of physical chemical techniques, e.g., optical rotatory dispersion, nuclear magnetic resonance, ultraviolet and infrared spectrophotometry. From these data the site of interaction of the metal can be correlated with its effect on the reactions of the nucleic acids.

Major Findings: A. Stereoselective reaction of platinum complexes with DNA. Complexes of platinum have been used to probe the mechanism by which metal ions bind to DNA and affect the mechanism of RNA synthesis (see also Report No. HD-AG 30). Platinum is extremely useful because it forms bonds which are much less labile than those of most other metal ions, so that intermediate stereoisomers are stable. Others have shown that

cis$[Pt(NH_3)_2Cl_2]$ is an effective antitumor agent, and that it inhibits cell division and DNA replication, while the trans complex is not effective. Important progress has been made in this laboratory to explain these phenomena. The cis complex forms two types of complexes with DNA that are not produced with the trans complex. One contains approximately one platinum per ten DNA nucleotide residues; its properties suggest a structure in which platinum atoms are bound to the exposed DNA bases, which are the same distance apart (3.4 Å) as the cis liganding positions of the platinum. The other contains one platinum atom per DNA residue; its properties suggest a structure (which is however not proved) in which platinum atoms, which form bonds with each other with 3.4 Å distances, are costacked with the DNA bases.

The 1:10 Pt/DNA nucleotide adduct is a manifestation of the most significant physical chemical differences exhibited by the cis and trans complexes in their interaction with DNA. The addition of small increments of the cis complex to DNA produces a marked enhancement of the ellipticity in the circular dichroism spectrum; the enhancement is maximal at the 1:10 ratio, and further incremental addition of the complex produces decreases in ellipticity. In contrast, the ellipticity of the adducts of DNA and the trans isomer decreases consistently with the addition of every increment of the platinum complex. The ellipticity does not increase when the cis complex is reached with denatured DNA, suggesting that the intact double helix is required for the reaction with platinum. The stoichiometry of the adduct of the cis complex with DNA can be explained if chelation of the platinum complex occurs to two adjacent bases only when a specific base sequence is present. We do not know at the moment which base sequence is involved.

$[Pt\ en\ Cl_2]$, which contains the chelating ethylenediamine group instead of two coordinated ammonia molecules, and which must therefore have the cis configuration, reacts with DNA in a manner identical to that of cis$[Pt(NH_3)_2Cl_2]$. Trans$[Pt(NH_3)_2Cl_2]$ reacts very differently, forming a 1:2 Pt/DNA nucleotide adduct, which presumably involves platinum as a crosslink between the DNA strands. These differences in reactivity of the platinum isomers result in marked differences in template activity, as discussed in Report No. HD-AG 30.

B. The higher stability of polydeoxynucleotides vs. polyribonucleotides toward denaturation by metal ions. Metal ions are able to distinguish between polyribonucleotides and polydeoxynucleotides by destabilizing single or double helical conformations of ribopolynucleotides much more readily than those of polydeoxyribonucleotides. Moreover, the ease with which the conformations of the polydeoxynucleotides are disorganized is sensitive to the nucleotide sequence. Thus Cu^{2+} ion, one of the most effective destabilizers of nucleic acid structure, has no effect on the poly dAT double helix, although it destabilizes DNA. Taken together with the previously determined greater susceptibility of RNA, compared with DNA, to cleavage by metal ions, it is apparent that DNA is much more able to withstand the various stresses to which metal ions subject the polynucleotide structure. Perhaps this is an advantage of DNA as a primary source of genetic information.

Copper ions are capable of disordering the helical structure of poly dA, but all other metals that destroy the poly rA helix are ineffective toward poly dA,

again demonstrating the greater stability of the polydeoxynucleotide structure.

C. Mechanism of destabilization of ordered structure of polynucleotides by metal ions. A combination of spectrophotometric, titration, and binding studies of the reaction of copper with oligonucleotides and polynucleotides has led to a mechanism of destabilization that involves crosslinks between phosphate and base groups. The high effectiveness of copper in disordering polynucleotide structure at neutral pH, where the free copper concentration is low, indicates that a hydroxylated species is responsible for the crosslinks. Titration studies indicate that this species contain two copper atoms and two hydroxyl groups. At high polymer concentrations the crosslinks are predominantly intermolecular, and as the polymer concentration is decreased, intramolecular crosslinks are preferred. These results indicate different mechanisms for the denaturation of polynucleotides at different concentrations.

D. The binding of copper(II) to transfer RNA. Metal ions are necessary to maintain the active conformation of t-RNA. It has been previously shown by nuclear magnetic resonance studies that Cu^{2+} ions are quite selective in binding to the bases of randomly coiled RNA. The binding of Cu(II) to tRNA, on the other hand, does not proceed according to base preference, but occurs in response to the conformational requirements of the molecule. It appears reasonable that metal ions fit into crevices of the tertiary structure so as to maintain the active native conformation.

When the reaction of Cu(II) with tRNA is studied in a DMSO-water mixture, in which the tRNA is denatured, the copper again exhibits preference for binding to the purines, and not to the pyrimidines.

Significance to Bio-medical Research and the Program of the Institute: The participation of metal ions in every aspect of genetic information transfer makes the study of the interaction of metal ions with the nucleic acids of major importance in understanding the perturbation of the genetic information that is presumably characteristic of the aging process.

Proposed Course of Project: It is intended to continue studies to determine the sites of binding of metal ions on nucleic acids, the conformational changes induced by such binding, and to correlate these effects with biological function. We have begun to determine the concentration of metal ions in biological materials, and the changes in these concentrations with age. We intend to determine changes in the permeability of cells to metal ions or metals complexed to small or large molecules, as a function of age.

Honors and Awards: None

Publications:

Rifkind, J. M., and Eichhorn, G. L.: Interaction of Metal Ions with Polynucleotides and Related Compounds. XXI. Metal Ions as Agents for the Stacking of Nucleotides. A Specific Interaction of Zinc(II) and Adenosine Monophosphate. J. Amer. Chem. Soc. 94: 6526-6534, 1972.

Berger, N. A., and Eichhorn, G. L.: Stereoselective Differentiation between Ribonucleosides and Deoxynucleosides by Reaction with the Copper(II) Acetate Dimer. Nature New Biology 95: 237-240, 1972.

Serial No. HD-GMA2
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Degradation of Nucleic Acids by Metal Ions

Previous Serial Number: Same

Principal Investigator: J. Butzow (30% time)
G. Eichhorn (5% time)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.55
Professional:	.35
Others:	.20

Project Description:

Objectives: (1) To render metal ion degradation of polynucleotides useful in the detection of nucleotide sequence and in selective degradation of ribonucleic acids. (2) To study the mechanism of the metal ion degradation reaction. (3) To utilize the ability of metal ions to degrade polynucleotides in the probe of the structure of biological macromolecules such as 5S-RNA.

Methods Employed: After brief exposure of 5S-RNA to zinc ion, the RNA is subjected to conditions favoring unfolding of its ordered structure to release parts of the chain between the zinc cleavages. The fragments are separated by electrophoresis through polyacrylamide gel under these conditions, and are detected in the gel by ultraviolet absorption scanning.

Major Findings: We have previously analyzed the positions of zinc cleavage in 5S-RNA, at the level of 1 phosphodiester bond broken for each RNA chain (120 nucleotide residues long), using introduction of ^{32}P-phosphate groups at the 5' hydroxyls into nonradioactive RNA. There were technical problems with this method, but the results were consistent with cleavage in sections of the RNA which are not base-paired. We have now directly investigated the size distribution of the fragments produced by zinc cleavage under reaction conditions which are such that the initial RNA conformation should not be significantly altered from its native state (as determined by optical rotatory dispersion). We find that reaction at 10 Zn/P and 40° will convert up

to 10% of the bulk of the RNA into a sharply moving fragment (or fragments) which is (are) of a chainlength between that of the undegraded 5S-RNA and "4S" or tRNA (about 80 nucleotide residues), as well as some material of the size range of "4S" RNA, before a heterogeneous gel profile results. We conclude that brief exposure of 5S-RNA to high concentrations of zinc ion can produce coherent cleavage and assume that this cleavage reflects kinks in the structure of the native form of the 5S-RNA.

Significance to Biomedical Research and the Program of the Institute: 5S-RNA is a component of the 50S subunit of the bacterial ribosome; it is the simplest ribosomal component and the one most readily capable of structural elucidation. In order to understand protein biosynthesis, the structure of the ribosome must be understood: the studies with zinc are designed to aid in this undertaking. The usefulness of zinc ion for selective degradation of RNA molecules is in itself of general technical biochemical interest.

Proposed Course of Project: On the basis of the currently reported results, we intend to probe the positions of zinc cleavage in the nucleotide sequence of 5S-RNA using uniformly ^{32}P-labelled RNA, with the methods of analysis of ^{32}P-labelled oligonucleotides already worked out by Sanger for this particular RNA.

Honors and Awards: None

Publications: None

Serial No. HD-GMA3
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Structure of Nucleoprotein, Ribosome, and Interactions of Components Related to Protein Synthesis

Previous Serial Number: Same

Principal Investigator: J. Butzow (70% time)
G. Eichhorn (20% time)
Y. Shin (60% time)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	2.10
Professional:	1.50
Others:	.60

Project Description:

Objectives: (1) To determine the conditions for the binding of proteins to nucleic acids, (2) to examine the mediation of this binding by metal ions, (3) to elucidate the structure of chromatin with the help of these model systems, (4) to understand how cellular differentiation and aging may be related to nucleoprotein structure, (5) to study the structure of the ribosome and its interaction with other substances engaged in protein synthesis.

Methods Employed: (1) The interactions of proteins and nucleic acids, and the effect of metal ions on these interactions are studied by optical rotatory dispersion, circular dichroism, spectrophotometry, to determine conformational changes, and by infrared and nmr techniques, to determine binding sites. (2) Models containing selected characteristics of chromatin are examined to determine which characteristics are responsible for chromatin function. (3) Conformational changes involved in the interaction of ribosomes, messenger RNA, transfer RNA, and other factors involved in protein synthesis are studied by means of optical rotatory dispersion and fluorescence.

Major Findings: A. Interactions of synthetic polypeptides with DNA, and the structure of chromatin. The histones that are bound to DNA in chromatin have been implicated in the regulation of gene expression. The various histone fractions are rich in basic amino acids, and contain clusters of lysine and arginine. It is therefore very useful to compare the interaction of polylysine and polyarginine with DNA to the interaction of lysine-rich and arginine-rich histones with DNA. Both synthetic polypeptides bind strongly to DNA and significantly stabilize it. They differ dramatically, however, in their effect on the conformation of DNA as demonstrated by optical rotatory dispersion (ORD). Polylysine produces an ORD curve with a large negative rotation at 290 nm, whereas polyarginine produces only a slight decrease in the magnitude of the ORD. Divalent metal ions influence the melting temperature of both types of DNA complexes, but they do not influence the ORD of the polyarginine complex, in contrast to major effects on the polylysine complex. The effects of polylysine on DNA resemble those of the lysine-rich histone I, and the similarity is augmented by introducing alanine residues into the polylysine(alanine - lysine copolymer). The effects of polyarginine resemble those of arginine-rich histone IV.

A comparison of the stability of the DNA complexes of polylysine, polyarginine, and polyornithine, using as criteria the melting temperature and the salt concentration required to break up the complex, reveals that basic polypeptides with long side chains form weaker links to DNA than shorter chain polypeptides. L-polylysine and D-polylysine form complexes with DNA of similar stability and properties but DL-polylysine forms a much less stable complex with a lesser effect on the conformation of DNA. All of these phenomena can be readily explained by the use of molecular models, and they enable us to comprehend the forces that bind histones in DNA.

DNA that is not bound to polypeptide components has a role in regulating the conformation of polypeptide-bound DNA. This observation could explain the fact that only part of the DNA in chromatin is covered by protein, while all of the chromatin is repressed toward in vitro transcription.

B. Physical chemical studies of the interaction of ribosome, messenger RNA, and transfer RNA. The ORD of salt-washed E. coli ribosomes and of mixtures of such ribosomes with poly(rU) and $tRNA^{Phe}$ were determined at $\sim 2^{o}$, pH 7.0, and .01 M Mg ion, at ribosome concentrations of the order of 10^{-3}M(P), and poly(rU) and $tRNA^{Phe}$ concentrations of the order of 10^{-4}M(P); no significant differences emerged between the ORD of the mixtures and the calculated combinations. Direct-difference determinations, under the same conditions except at ambient temperature of 25-27^{o}, unequivocally showed no significant differences, and so demonstrate that, under these particular conditions, no conformational differences measurable by ORD exist for these combinations. Experiments utilizing the fluorescence of the Y nucleotide of yeast $tRNA^{Phe}$, which is located in the anticodon loop, were conducted with the same system and have also shown no net changes so far. Thus it appears that the components of the very simple system consisting of ribosome, messenger RNA, and transfer RNA do not influence each other's conformation, at least as detectable by these methods. If it can be shown that the addition of other factors does produce conformational changes, the inducers of these changes will be readily identified.

Significance to Bio-medical Research and the Program of the Institute: An understanding of the structure of chromatin is required to understand the mechanism of gene regulation, which is of direct consequence to the study of development and aging. The ribosome is the locus of protein biosynthesis, and the agency or locus of much of its control. Our physical studies are designed to provide direct information about structures and interactions which are intimately involved in the synthesis process and its ribosomal control. If we are able to understand these interactions, it will then be possible to determine how they may change with age.

Proposed Course of Project: We expect to continue to analyze the interaction modes of DNA with the protein constituents of chromatin by comparison of the interaction with DNA of the chromatin proteins with synthetic polypeptides. We shall study the effect of translational cofactors on the conformation of the ribosome in the presence of message and both charged and uncharged transfer RNA.

Honors and Awards: None

Publications: None

Serial No. HD-GMA5
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Polymers as Biological Reagents

Previous Serial Number: Same

Principal Investigator: J. Pitha (90% time)
H. Chou (100% time) (E.O.D. 1/2/73)

Other Investigators: D. Lowy
N. Teich
P. Pitha

Cooperating Units: Laboratory of Viral Diseases, N.I.H.
Johns Hopkins University
Baltimore City Hospitals

Man Years:

Total:	2.40
Professional:	.40
Others:	1.00

Project Description:

Objectives: The program focuses on the effects of synthetic polymers on human cells grown in tissue culture and on the interaction of polymers with viral systems (animal viruses). The main class of macromolecules studied consists of the vinyl analogs of nucleic acids of the general formula $(-CH\ Base - CH_2)_n$; furthermore a series of compounds with polynucleotide bound to protein or to polysaccharide has been investigated. The ultimate objectives are to determine which synthetic polymers can be used pharmaceutically, to study viral infection in senescent cells and the possibility of its prevention by polymers, and finally, to determine whether the uptake of foreign macromolecules by cells changes with the age of the cells.

Methods Employed: In the study a very broad range of chemical and biological methods has to be used and several laboratories are involved in collaborating on this project. The majority of the chemical synthesis, some *in vitro* work with enzymatic systems (replicase) and the uptake of dextran by cells grown in tissue culture is done at the Gerontology Research Center. The study of the replication of murine leukemia virus is done in co-work with Drs. D. Lowy and N. Teich from the Laboratory of Viral Diseases, N.I.H., Bethesda. The rest of the work involving viruses is done in collaboration with Dr. P. Pitha, Department of Medicine, Johns Hopkins

University. In vitro protein synthesis is studied with Drs. F. Reynolds and D. Gruenberger from Columbia University.

Major Findings: A. In vitro synthesis of proteins. The effects of poly(vU) on in vitro protein synthesis directed by mRNA has been investigated. The amount of protein formed was found to decrease in different degrees, depending on the nature of the particular RNA.

B. Polymers and living cells. Vinyl analogs of nucleic acid were found to be taken up by cells in tissue cultures. Polyvinyluracil at high concentration is toxic to rapidly growing cells; polyvinyladenine (or the uracil analog at much lower concentration) does not interfere with cell multiplication and the synthesis of nucleic acids and proteins.

C. Polymers and viral replication. Polyvinyluracil and polyvinyladenine as well as the corresponding polynucleotides polyuridylic and polyadenylic acid inhibit acute murine leukemia virus infection in mouse embryo cells, but do not significantly inhibit the replication of Sindbis and vesicular stomatitis viruses. These polymers were most effective when added during an early stage of murine leukemia virus replication. The inhibition of the leukemia virus appears to be related to an inhibition of an RNA-dependent DNA polymerase.

D. Polymers and induction of interferon. The effects of different chemical modifications of the complex of polyinosinate and polycytidylate on its antiviral activity have been studied. Using base substitutions which conserve the double helix of the complex, it was found that antiviral activity and interferon induction are more critically influenced by substitution in the inosinate than in the cytidylate strand. The rigorous steric requirements (as embodied by the poly I·poly C structure) for antiviral protection by polynucleotides are significantly decreased when aggregated interferon inducers are used. Significant protection against virus infection was achieved by complexes of various polybases, such as polylysine or DEAE Dextran, with polyinosinate; introduction of cytosine residues covalently bound to these polybases in the complex is without specific influence. Furthermore, the complex of polyinosinate with polycytidylate was attached to solid carriers, such as Sephadex or cellulose, which may be suspended in the medium over the cell monolayer; it was also attached to surfaces which may be overgrown by the cell monolayer. Interferon production and antiviral protection achieved by these inducer systems were measured and it was found that such anchored poly IC conveyed antiviral protection. However, in all systems studied, some release of polynucleotide from the carrier was observed and thus the results do not prove that mere contact of inducer with the external membrane is sufficient for induction of interferon.

E. Polymers and senescent cells. A previously prepared series of radioactive dextran macromolecules of differing molecular weights has been used to study whether there is any change in the uptake of foreign macromolecules with aging of cells in tissue culture. No particular age differences have been detected but results are not yet final.

F. Senescent cells and viral infection. An extensive study of abortive infection of senescent cells by vesicular stomatitis virus and its prevention

has led to the following results: 1) Senescence of host cells is not detrimental to viral replication with the exception of a brief period before cellular death. 2) Cellular viral defenses are functional throughout the life span of the cells up to the moribund state but the amount of antiviral agent required increases with age. 3) In senescent cells polynucleotide interferon inducer produces the antiviral state faster than treatments with interferon itself.

Significance to Bio-medical Research and the Program of the Institute: Synthetic compounds of high molecular weight differ profoundly from low molecular weight compounds in their interaction with living cells. They are taken up by a different mechanism, the efficiency of which seems to depend more on the growing stage of the cell than on the age of the cell. Another difference is in their degradation in cells; synthetic macromolecules have a much longer life span since they are not susceptible to the usual cellular degradative processes. Both these differences have practical importance only when polymers with a selective biological action can be designed and synthetized. Vinyl analogs of nucleic acids do show selectivity thus pointing to their potential use as pharmaceuticals.

Proposed Course of the Project: The present results show that synthetic polymers can elicit a selective biological effect, in a manner similar to that of low molecular weight pharmaceuticals. By directed synthesis and study of the basic biological effects of polymers it is hoped to gain the knowledge necessary for the design of more potent preparations.

Honors and Awards: None

Publications:

Reynolds, F., Grunberger, D., Pitha, J., and Pitha, P. M.: Inhibition of Cell-Free Protein Synthesis by Poly(9-vinyladenine), Poly(1-vinyluracil), and the Corresponding Vinyl Copolymer. Biochemistry 11: 3261-3266, 1972.

Pitha, P. M., and Pitha, J.: Nonenzymatic Synthesis of Oligoadenylates on Template lacking Steric Regularity. Nature New Biology 240: 78-80, 1972.

Pitha, P. M., Teich, N. M., Lowy, D. R., and Pitha, J.: Inhibition of MLV Replication by Polyvinyluracil and Polyvinyladenine. Proc. Natl. Acad. Sci. 70: 1204-1208, 1973 .

Pitha, P. M., Teich, N. M., Lowy, D. R., and Pitha, J.: Inhibition of Leukemia Virus Replication by Vinyl Analogs of Polynucleotides. In Wells, R. D., and Inman, R. B. (eds.): DNA Synthesis In Vitro. University Park Press, in press.

Serial No. HD-GMA6(1)
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Mispairing of Nucleotide Bases

Previous Serial Number: Same

Principal Investigator: G. Eichhorn (10% time)
J. Pitha (10% time)
E. Tarien (30% time)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.70
Professional:	.50
Others:	.20

Project Description:

Objectives: (1) To determine the conditions under which mispairing of the complementary bases occurs between polynucleotide strands in order (2) to understand the ways in which misreading of the genetic code can occur in order (3) to understand how errors that could lead to the aging of organisms can occur in the apparatus for the transmission of genetic information.

Methods Employed: It is known that metal ions of the wrong type or in the wrong concentration can bring about the misreading of the genetic code in protein synthesis, resulting in the production of proteins containing incorrect amino acid sequences. Such errors can arise through mispairing of bases in codon-anticodon recognition. The following hypothesis is devised to account for mispairing. It is known that high concentrations of divalent ions stabilize the interaction of polynucleotide strands. In the absence of the divalent metal ions these interactions are therefore relatively unstable and require base complementarity; in the presence of these ions the interactions are stabilized to such an extent that sensitivity to complementarity is lost, and mispairing then occurs.

This hypothesis can be tested by studying the binding of homopolynucleotides to heteropolynucleotides containing one base that is complementary and one base that is not complementary to the base in the homopolymer. The extent of mispairing is measured by (a) stoichiometric studies of the

reaction between the two polynucleotides and (b) by nuclear magnetic resonance studies that detect only non-hydrogen bonded bases.

Major Findings: A. NMR studies on mispairing. It had been previously demonstrated that divalent ions can induce the mispairing of nucleotide bases. Nuclear magnetic resonance studies had shown that magnesium ions induce mispairing in the system poly(A,U):poly(I). It has now been shown in the same way that magnesium ions induce mispairing in the systems poly(U,C):poly I and poly(A,C):poly I. In every case NMR lines that are detectable in the absence of magnesium become broadened when magnesium is added, thus demonstrating the interaction of non-complementary base pairs in these systems as well. These studies show that the mispairing effect is general, and not restricted to any type of base pairing.

B. NMR sequence studies. As a corollary to the mispairing studies described above, we have learned that the NMR peaks associated with a given nucleotide base may be shifted in different degrees by association with other nucleotide bases in the vicinity. Thus the adenine peaks in poly(A,C) appear to split into two groups that appear to be associated with adenines surrounded by other adenines and adenines surrounded by cytosines. This discovery may furnish a technique for the study of base sequence and will be pursued further.

Significance to Bio-medical Research and the Program of the Institute: The accumulation of errors that would be produced during protein synthesis by mispairing of nucleotides of the kind being studied would lead to deleterious effects in an organism, according to one of the more popular aging theories. Thus the study constitutes a chemical model for a theory of aging.

Proposed Course of Project: We intend to determine whether metal ions in biological systems will produce the kind of errors that are induced in these model systems. We hope to investigate the usefulness of the NMR method in the determination of base sequence.

Honors and Awards:
G. Eichhorn was elected Fellow of the Gerontological Society

Publications: None

Serial No. HD-AG17(8)(c)
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Relation of Structure and Function in Hemoglobin

Previous Serial Number: Same

Principal Investigator: J. Rifkind (60% time)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	.70
Professional:	.60
Others:	.10

Project Description:

Objectives: (1) To study the binding of ligands to hemoglobin, and the role of the protein in controlling this function. (2) To study the mechanisms for maintaining hemoglobin in its functional form. (3) To study the mechanisms involved in regulating the transport of oxygen to the tissues.

Methods Employed: Hemoglobin is purified by gel filtration. The purified hemoglobin is compared to hemolyzed cells with respect to the rate of autoxidation, the concentration of various metal ions and other red cell components, and the Cu(II) activity. The oxidation is also investigated with and without the addition of various substances such as metal ions, complexing agents, and oxidizing agents. The relationship between the oxidation of hemoglobin and the ligands bound to hemoglobin are investigated by removing or changing the ligands. The direct interaction of copper with hemoglobin is studied by EPR spectroscopy.

Major Findings: A. The involvement of copper in the autoxidation of hemoglobin. Oxygen only binds to reduced hemoglobin. Therefore, the autoxidation of functional hemoglobin decreases its oxygen binding capacity and thereby its ability to transport oxygen to the tissues. Our previous studies on the stabilizing effect of metal complexing agents and the specific catalytic oxidation of added copper suggested the involvement of copper in the autoxidation of hemoglobin.

The validity of this hypothesis and the importance of this effect has been established by measurements of the total copper and Cu(II) activity in

hemolyzed cells and purified hemoglobin preparations. Hemolyzed cells contain approximately one copper atom for every 1000 hemes. This concentration of copper is more than adequate to produce appreciable catalytic oxidation. When the hemoglobin is purified by gel filtration, all the copper chromatographs together with the proteins, and the ratio of copper to hemoglobin remains about the same. The autoxidation in both hemolyzed cells and purified hemoglobin can therefore be attributed to copper originating in the erythrocyte. The dominant role of copper in the autoxidation of hemoglobin is suggested by the excellent correlation under various conditions of the rates of autoxidation with the Cu(II) activity which must determine the extent to which copper is interacting with hemoglobin. About half of the copper can be separated from the proteins, lowering the rate of autoxidation, by adding a complexing agent to the hemolyzed cells prior to gel filtration. This result suggests that only half of the erythrocyte copper is actually involved in catalyzing the autoxidation. The remaining copper is probably coordinated to erythrocuprein (superoxide dismutase) from which copper cannot be removed by complexing agents at neutral pH.

All investigators working with purified hemoglobin have found that almost anything you do with hemoglobin seems to increase the rate of autoxidation. We have also been plagued by this problem. However, the analysis of copper indicates that these problems can be related to the ubiquitous presence of trace amounts of copper on glassware, equipment and in solutions. The strong affinity of hemoglobin for copper tends to concentrate the copper in the hemoglobin solutions. Our studies have only been possible by extreme caution in minimizing copper contamination. When this is done the hemoglobin remains surprisingly stable with respect to autoxidation.

B. The mechanism for the catalytic oxidation of hemoglobin by copper. Considering the significance of the oxidation of hemoglobin by copper we are attempting to elucidate the details of the mechanism. By studies involving deoxyhemoglobin, in the absence of oxygen, it has been possible to show that copper is able to directly oxidize the iron of hemoglobin. The role of oxygen for the catalytic effect is, therefore, probably to regenerate the Cu(II) ion from the Cu(I) ion formed when copper oxidizes hemoglobin. We have also been able to show that the sixth ligand must be removed from hemoglobin prior to the oxidation. Therefore, the rate of oxidation by copper is greatest for deoxyhemoglobin where no sixth ligand is present. In the presence of a sixth ligand the rate is actually retarded by higher ligand concentrations or by replacing oxygen by carbon monoxide which dissociates from hemoglobin much more slowly.

Finally the binding of copper to hemoglobin seems to be required for the oxidation. Thus the strong affinity of hemoglobin for copper is seen by very low copper activities in the presence of hemoglobin. EPR studies also indicate that there is very little free copper present when copper is added to hemoglobin. The involvement of the binding for the oxidation is consistent with the apparent difference in the EPR spectrum for copper bound to reduced and oxidized hemoglobin. Such a change in the nature of bound copper is expected if the binding is to facilitate oxidation. The unique role of copper can possibly be explained by a combination of the right oxidation potential, to oxidize hemoglobin and be regenerated by oxygen, and the strong binding to

a particular region of hemoglobin where electron transfer between the iron and the copper is facilitated.

C. The regulation of oxygen utilization in the muscle. Oxygen storage and transport in the muscle involves myoglobin. This protein reversibly binds oxygen, and has a structure very similar to the individual hemoglobin subunits. In a collaborative study with Dr. Lumry (Univ. of Minnesota) we are studying a possible mechanism for the regulation of oxygen release by myoglobin. In the preparation of myoglobin it has been found that a small ionic substance bound tightly to myoglobin, which can be oxidized and reduced, influences the oxygen affinity of myoglobin. We have found that copper chromatographs together with this regulatory substance suggesting that the substance is perhaps copper or another substance which binds copper strongly.

Significance to Bio-medical Research and to the Program of the Institute: The physiological role of hemoglobin is to transport oxygen from the lungs to the cells. The efficient uptake and release of oxygen requires cooperative oxygen binding and the proper regulation of the oxygen affinity. It is also necessary to limit the oxidation of hemoglobin in order to maintain an adequate concentration of functional hemoglobin. These studies thus help to elucidate a vital function of organisms. The aging process can involve changes in the ability of the organism to transport oxygen to certain tissues.

Proposed Course of Project: (1) We plan to continue our studies on the autoxidation of hemoglobin in the erythrocyte and in purified systems. The possible role of superoxide dismutase will be investigated. (2) The mechanism for the oxidation of hemoglobin by copper will be further studied. (3) Numerous clinical conditions, requiring more effective oxygen transport, result in increases in the level of 2,3-DPG which decreases the oxygen affinity of hemoglobin facilitating the release of oxygen to the tissues. An increase in the level of 2,3-DPG for human subjects above age 50 has also been reported. This finding suggests that a more effective release of oxygen from hemoglobin may be required to overcome certain degenerative changes occurring with age. We plan to test this hypothesis by measuring levels of 2,3-DPG in whole erythrocyte as a function of the age of the organism and correlating these levels with the oxygen affinity. (4) We also will attempt to change the level of 2,3-DPG in erythrocytes *in vitro* as well as *in vivo* in order to investigate the possible effect of these changes on particular aging processes.

Honors and Awards: Dr. Rifkind was invited to present a seminar to the Division of Hematology, Johns Hopkins University School of Medicine, Baltimore, Md.

Dr. Rifkind was invited to present a seminar to the Heme-Protein group at the National Institutes of Health, Bethesda, Md.

Publications:

Rifkind, J. M.: The Autoxidation of Horse Hemoglobin: The Effect of Glutathione. *Biochim. Biophys. Acta* 273: 30-39, 1972.

Serial No. HD-AG30
1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Molecular Chemistry Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: The Function of Metal Ions in Enzymatic Reactions

Previous Serial Number: Same

Principal Investigator: P. Clark (100% time)
G. Eichhorn (30% time)
J. Froehlich (100% time) (E.O.D. 8/1/72)

Other Investigators: None

Cooperating Units: Baltimore City Hospitals

Man Years:

Total:	2.30
Professional:	2.30
Others:	

Project Description:

Objectives: (1) To elucidate the mechanism by which metal ions activate or inhibit enzymatic reactions. (2) Specifically, to elucidate the role of bound metal ions in transcription by studying their mode of binding to RNA polymerase, and their role in substrate recognition, binding and message synthesis.

Methods Employed: RNA polymerase obtained from E. coli contains approximately 2 gram atoms of zinc/mole of core enzyme following prolonged dialysis with chelating agents. The weak polarizability and diamagnetism of zinc make it an unsuitable probe of site conformation. The unpaired electrons of the metals of the first transition series provide a sensitive means with which to monitor (1) the local geometry of the binding site (2) the proximity of neighboring paramagnets and (3) the accessibility of counterions (Cl^-, Br^-) to the metal using the techniques of electron paramagnetic resonance spectroscopy and pulsed nuclear magnetic resonance spectroscopy. The effective use of these probes requires preservation of the native structure and partial retention of enzymatic activity. The kinetic properties of the substituted enzyme can be used to assess the effects of metals on the initiation, polymerization, and termination of message. Preparation of the substituted enzyme will be attempted by (1) metal exchange in the presence of high concentrations of the desired probe (2) removal of zinc by chelation followed by stoichiometric addition of the paramagnetic probe in

a zinc-free medium. Atomic absorption spectrophotometry and enzymatic activity will be used to follow the course and extent of substitution.

A new method has been developed for determining whether a double helix is enzymatically cleaved by simultaneous attack on both strands of the helix, or by attack on only one strand at a time. The method consists of an analysis of the nucleotide content of the stabilized fraction.

Major Findings: A. RNA polymerase. Very tight binding of zinc is indicated by the failure to remove $>$ 25% of the bound metal after 8 days of dialysis vs. 5mM orthophenanthroline. It has however been demonstrated that the zinc can be displaced by Mg^{2+}, Co^{2+} and Mn^{2+} with the retention of full activity, and by Cu^{2+} with 50% activity. The copper enzyme contains 2-4 Cu^{2+} ions per enzyme. Its electron spin resonance spectrum contains a dipole interaction signal which reveals that two copper atoms bound to the enzyme are 5-6 Å apart. Cu^{2+} ions the same distance apart are also present in an enzyme to which manganese had been bound and from which it was then removed. Thus Cu^{2+} ions readily attach themselves to a strong binding site which may be the intrinsic metal binding site of the enzyme.

The cobalt enzyme was subjected to kinetic studies which demonstrated kinetics similar to those of the native zinc enzyme. This behavior is in direct contrast to the effect on the enzyme of free cobalt (which had not displaced zinc), which prolongs the initiation phase but enhances the rate and extent of polymerization.

As reported in HD-GMA-1, cis$[Pt(NH_3)_2Cl_2]$ forms a 1 Pt/10 DNA nucleotide adduct with DNA. Because of the biological activities of this complex it was decided to evaluate its effect on the ability of DNA to act as a template for RNA synthesis. The complex was found to confer little or no change in the initiation process of RNA polymerase, but a dramatic inhibition was observed in the polymerization phase of the enzymatic process. $[Pt\ en\ Cl_2]$ had an identical effect. In both cases polymerization was inhibited by approximately 90%. These experiments were carried out by first incubating the DNA with the platinum complex for 24 hours to insure the formation of the adduct. When other experiments were carried out without such pre-incubation, so that no binding of the platinum complex to the DNA occurred, a 50% inhibition of the polymerization was nevertheless observed. The most likely interpretation of these experiments is that the platinum complex inhibits transcription in two ways, by binding to the DNA as well as by binding directly to the enzyme.

B. Mechanism of DNA cleavage. Porcine spleen acid DNAse is known to cleave DNA by operating simultaneously the phosphodiester bonds of both nucleotides of a Watson-Crick base pair. The new method for determining whether cleavage occurs by simultaneous scission in both chains or by scission of individual strands consists of analyzing the base content of soluble oligonucleotides. If the analyses add up to complementarity, there must have been simultaneous scission. This is in fact what was found with the acid DNAse, indicating that the method does work.

When applied to bovine pancreatic DNAse I, the base analyses did not display complementarity, when the enzyme had been activated with several metal ions.

It is concluded that with this enzyme only one strand is cleaved at a time, thus reversing conclusions previously reached by more devious techniques.

C. The binding of nickel ions in serum. In a collaboration with F. W. Sunderman of the University of Connecticut, it has been demonstrated that Ni^{2+} ions in serum are bound principally to aspartic acid in a 2:1 complex with apparently two carboxyl groups and an amino group coordinated to the nickel. This study is of some importance as a beginning in determining where metal ions introduced into a living organism are attached.

Significance to Bio-medical Research and the Program of the Institute. The processes of RNA synthesis and DNA degradation are of fundamental importance to an understanding of cellular processes. Metal ions are required for these processes and variations in their effects may be of significance in the deleterious changes accompanying aging.

Proposed Course of Project: We shall continue to pursue the elucidation of the mechanism of transcription as well as the mechanism of enzymatic DNA degradation, as outlined in the Methods section.

Honors and Awards:
G. Eichhorn was invited to present an address at a Symposium on the "Role of Metal Ions in Biological Systems", at Argonne, Illinois, November, 1972.

G. Eichhorn was invited to present a lecture at a Symposium on "Bioinorganic Chemistry" in Oneonta, New York, May, 1973.

G. Eichhorn was invited to present a lecture at a Symposium of the American Chemical Society on "Bioinorganic Chemistry", Kalamazoo, Michigan, June, 1973.

G. Eichhorn was invited to lecture at a Gordon Conference Symposium on Bioinorganic Chemistry, New Hampton, N. H., August, 1973.

G. Eichhorn was asked to organize and to chair a Symposium on the "Regulation, Transport and Role of Inorganic Elements in Living Systems" at the American Chemical Society meeting in Chicago, Illinois, August, 1973.

G. Eichhorn was invited to be a participant in the NATO Science Committee Conference on Catalysis, Cagliari, Sardinia, December, 1972.

G. Eichhorn was invited to present seminars at various Universities.

J. Froehlich was invited to present a seminar at Johns Hopkins University, Baltimore, Md. February, 1973.

Publications:
Eichhorn, G. L. (Editor): Inorganic Biochemistry, Vol. I, 610 pp. Elsevier Publishing Co., in press.

Eichhorn, G. L.: Binding of Nickel to Biological Substances. In Sunderman, F. W. (ed.): Nickel, Man and his Environment. National Academy of Science, in press.

Eichhorn, G. L.: Effects of Nickel on Enzymatic Activities. In Sunderman, F. W. (ed.): Nickel, Man and his Environment. National Academy of Science, in press.

Serial No. HD-GMA 7

1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Section on Intermediary Metabolism
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Cellular Regulation of Intermediary Metabolism

Previous Serial Number: HD-AG19(8) and HD-AG20(8)

Principal Investigators: Bertram Sacktor
Bernard Bulos
Walter Greenhouse
Shri Shukla
Peter Chiang

Other Investigators: Stephen O'Brien (Guest Worker) 7/1/72 - 9/30/72

Cooperating Units: None

Man Years:

Total:	7.2
Professional:	4.5
Others:	2.7

Project Description:

Objectives: The broad objective of this project is to describe the general mechanisms that regulate intermediary metabolism in animals, including man. These mechanisms range from the simplest types of control that involve the intrinsic properties of enzymes affecting the rates of reactions to those of increasing complexity that comprise the action of modulators on regulatory and allosteric enzymes; genetic repression and derepression; selective separation, sequestration and release of ions, metabolites and enzymes by intracellular membranous organelles; steady state and transient fluxes of intermediates; and the actions of endogenous and exogenous chemical and physical modifiers on entire metabolic pathways and coordinated systems. The development of this scientific data and theory is focused on those physiological functions that may show significant alterations with development and aging and, thus, are essential to the understanding of the molecular processes involved in development and aging.

Methods Employed: Metabolic pathways are studied with purified enzymes, isolated organelles, cell free systems, cell cultures, slices of tissues, perfused organs and intact animals. Powerful tools of analysis of metabolites, measurements of enzyme activities, and isotope tracer techniques are employed, as appropriate. Computer analyses of kinetic models are used as required.

Major Findings: A. Control of Krebs cycle activity in mitochondria. Extensive studies on the control of pyruvate oxidation in mitochondria from blowfly flight muscle, and confirmed with mitochondria from rabbit heart muscle, have led to a novel mechanism for the regulation of Krebs cycle activity. Both ADP and inorganic phosphate are required for the uncoupled (FCCP) oxidation of pyruvate as well as for the coupled oxidation. The FCCP-stimulated oxidation is markedly inhibited by physiological levels of Ca^{2+}. The apparent K_i = 10 μM. The electron transport chain of the mitochondria is fully functional in the presence of Ca^{2+} since the oxidation of α-glycerol-phosphate is not inhibited, indicating control at the dehydrogenase level. Kinetics studies show that the Ca^{2+} inhibition is uncompetitive with respect to ADP and FCCP, but complex, and noncompetitive with respect to pyruvate. The results of these and other studies suggest that pyruvate oxidation and Krebs cycle activity is limited by NAD-dependent isocitrate dehydrogenase activity and Ca^{2+} plays a role in its regulation.

B. Control of pyruvate dehydrogenase activity: Interconversion of active and inactive forms. In experiments to determine the mechanisms of regulation of the mitochondrial pyruvate dehydrogenase complex, it has been observed that the enzyme complex from blowfly flight muscle exhibits Michaelis-Menton kinetics with respect to pyruvate and NAD, but shows sigmoidicity with CoASH. A Hill coefficient of 1.5 is obtained. The apparent Km values are: 0.21 mM for pyruvate, 39 μM for CoASH, and 0.28 mM for NAD. The Km value for pyruvate is similar to the Km value for State 3 oxidation of pyruvate in intact mitochondria, and approximates the concentration of pyruvate found in the muscle, *in vivo*. The concentration of CoASH in blowfly flight muscle has been determined to be 35 nmoles/g wet wt. Acetyl CoASH is a potent competitive inhibitor with respect to CoASH. Both ATP and GTP are non-competitive inhibitors with respect to pyruvate. Activity of the complex is influenced by the Mg^{2+} and Ca^{2+} concentrations and by the presence of chelating agents. CoASH forms a chelate with Mg^{2+}. A stability constant of 5,000 M^{-1} is obtained for the chelates of Mg-CoASH and Mg-thiamine pyrophosphate.

It has been found that control of pyruvate dehydrogenase complex activity in mitochondria from blowfly flight muscle, and confirmed with mitochondria from rabbit heart, is mediated additionally by the interconversion of the complex from a phosphorylated (inactive) form to a dephosphorylated (active) form. In flight muscle, pyruvate dehydrogenase kinase, which converts the active form to the inactive form requires ATP and Mg^{2+}. By Hill plot analyses, the apparent Km for ATP is 0.3 mM and the coefficient of interaction is between 2 and 3. Both pyruvate and inorganic phosphate are non-competitive inhibitors of the kinase whereas AMP is a competitive inhibitor. Changes in the concentrations of these effectors in the muscle *in situ* during rest and exercise are in the range consistent with their modulation of enzyme activity *in vivo*. Atractyloside, an inhibitor of adenine nucleotide translocation in mitochondria, does not block kinase activity. This may suggest that ATP is accessible to the kinase external to the atractyloside barrier. The phosphorylated pyruvate dehydrogenase is dephosphorylated and, thus, activated by a specific phosphatase. The phosphatase is inhibited by F^- and chelating agents and is activated by physiological concentrations of Mg^{2+} and Ca^{2+}.

C. Bioenergetics of mitochondria from flight muscle of aging blowflies. A correspondence between the decline in the ability of aged blowflies to fly and in the maximally stimulated respiratory rate (State 3) and respiratory control ratio of flight muscle mitochondria was reported previously. The biochemical mechanism and locus of this decrement with senescence has been investigated further by examining the bioenergetic properties of mitochondria from mature and aged flies for rates of oxidation during coupled oxidative phosphorylation, activities of the dehydrogenases, concentrations of components of the electron transport chain, phosphorylation of ADP, and the coupling mechanism, itself. Evidence, to date, indicates that the specific activities of α-glycerolphosphate and pyruvate dehydrogenases are unaltered by age. Similarly, the concentrations of cytochromes a, a_3, b, and $c+c_1$ are the same for both age groups. ATP synthetase, measured as ATPase, is unchanged with senescence. In contrast, the uncoupled oxidation of substrates is significantly decreased with aging, showing the 30-40% decrement between mature (7-10 days) and aged (31-33 days) as seen previously for the maximally stimulated coupled rate of respiration. These observations narrow the possible loci of the defect in aging to the bioenergetic system between the primary dehydrogenase and the FCCP-sensitive uncoupling reaction.

D. Genetic regulation of enzyme activity. The malate dehydrogenases of Drosophila melanogaster have been resolved into a cytoplasmic form (cMDH), and a mitochondrial matrix form (mMDH). Three isozymes of cMDH have been detected by starch gel electrophoresis and acrylamide gel isoelectric focusing. The probable structural gene for cMDH (Mdh-1) has been mapped genetically by allozyme variants to II-35±3, and cytologically by monitoring gene dosage in segmental aneuploids to between 28D and 29F on II-L of the Drosophila salivary gland chromosome map. The structural gene for mMDH is neither identical to nor in the near chromosomal proximity of Mdh-1. Nevertheless, the two enzymes exhibit markedly similar properties with respect to: (1) catalytic activity, (2) pH optima, (3) pH optimum shift in response to different ionic environments, and (4) molecular weight as determined by sucrose density gradient sedimentation.

A method for detecting possible structural genes in Drosophila melanogaster based on gene dosage dependency has been developed. By making 30 crosses between Y-autosome translocations, and an attached 4 cross, it is possible to produce large duplications (approximately 150 salivary gland chromosome bands in length) for every autosomal region with the exception of 83DE. The usefulness of the technique has been demonstrated by dosage dependency of three known gene-enzyme systems: α-glycerophosphate dehydrogenase-1, alcohol dehydrogenase and malate dehydrogenase. A screen for genes affecting two enzymes localized on the inner membrane of the mitochondrion, α-glycerophosphate oxidase (αGPO) and succinic dehydrogenase (SDH), produces a dosage sensitive region in each case. Region 50C-52E affects αGPO activity and region 28D-29F affects SDH activity. The latter region also includes the malic dehydrogenase-1 gene.

E. Molecular organization, biogenesis and turnover of mitochondrial membranes. The molecular organization of rat liver mitochondrial membranes is being studied by testing hypotheses on the mechanisms of mitochondrial turnover, e.g., by a dynamic process in which the membranes are assembled

from multiple components, each turning over at an unique rate, or by a process of replication and degradation as a single unit. Previous studies have demonstrated the utility of altering mitochondrial function and perturbing the mitochondrial membranes by changing the thyroid state of the animal. We have now found that during the transition from the hypothyroid state (thiouracil fed for 4 weeks) to the thyroid repleted state (a single injection of 20 μg T_3/100 g body wt) several enzymes, selected because they represent specific markers for intracellular membranes, behave in distinct fashion. For example, in confirmation of previous findings, the specific activity of α-glycerolphosphate dehydrogenase, an enzyme localized on the outer surface of the inner mitochondrial membrane, is decreased in the hypothyroid state to about 20% that in the euthyroid animal. In the T_3 repleted state, the specific activity is increased over 400% within 40 hours. In contrast, succinic dehydrogenase, another marker enzyme for the inner membrane, but believed to be located on the inner surface, responds differently. The specific activity of succinic dehydrogenase is increased 150% by hypothyroidism. T_3 repletion does not cause a return to euthyroid levels by the first 40 hrs, however. Monoamine oxidase, a marker for the outer mitochondrial membrane, shows a 2-fold increase in specific activity in the TU-fed rats and T_3-repletion causes a fall in specific activity to euthyroid levels. However, when mitochondria are subfractionated, the specific activities of the enzyme in the outer membrane from mitochondria of TU-fed and T_3-repleted animals are essentially the same. Therefore, these findings suggest that the changes in the specific activities of monoamine oxidase seen in whole mitochondria represent changes primarily in the protein content of the inner membrane, with the composition of the outer membrane remaining fairly constant. The enzymatic components of the microsomal membrane are also differently affected by the thyroid state. For example, the specific activity of glucose-6-phosphate dehydrogenase varies with the thyroid state but the specific activity of the rotenone-insensitive NADH-cytochrome c reductase does not. The phospholipid content of the different subcellular and submitochondrial fractions have also been examined with respect to the thyroid state. No significant changes are found in total phospholipid nor phosphatidyl ethanolamine, phosphatidyl choline and diphosphatidyl glycerol in the different membrane fractions. From these findings, the following tentative conclusions are indicated: (1) Protein is incorporated in the inner membrane of mitochondria independently of a change in the outer membrane; (2) A specific protein can be incorporated into the inner membrane without simultaneously incorporating other protein constituents of the inner membrane; (3) The depletion and repletion of a specific protein of the inner membrane is accompanied by corresponding changes in the phospholipid composition; and (4) Changes in membrane composition occur by additions and subtractions from a pre-existing mosaic structure which remains functional throughout the perturbation.

Significance to Bio-medical Research and the Program of the Institute: The acquisition of basic knowledge on the biochemical mechanisms regulating the intermediary metabolism of animals is of crucial importance in understanding the cellular processes fundamental to the function of physiological systems. This information is essential for the better understanding of the changes that occur in the chemistry of the body during development and aging.

Proposed Course: Mechanisms of the regulation of cellular metabolism will remain an area of intense investigation. Particular emphasis will be given to the control of bioenergetic pathways in mitochondria. Work in this area is immediately relevant to the determination of the basis for the decrement in excitation-contraction-bioenergetic coupling that is found during the aging process.

Honors and Awards:

Dr. Sacktor was invited to lecture at a Gordon Conference Symposium on The Biochemistry of Aging, Santa Barbara, California.

Dr. Sacktor was invited to present a lecture and lead a discussion on Cellular and Molecular Aspects of Aging: Mitochondrial Processes at Temple University, Philadelphia, Pennsylvania.

Dr. Sacktor was invited to present a seminar at Ohio State University, Columbus, Ohio.

Dr. Greenhouse was invited to present a seminar at The Johns Hopkins University, Baltimore, Maryland.

Dr. Sacktor was elected Fellow of the Gerontology Society.

Publications:

Bulos, B., Shukla, S., and Sacktor, B.: Bioenergetic properties of mitochondria from flight muscle of aging blowflies. Arch. Biochem. Biophys. 149: 461-469, 1972.

Bulos, B., Shukla, S., and Sacktor, B.: Effect of thyroid hormone on respiratory control of liver mitochondria from adult and senescent rats. Arch. Biochem. Biophys. 151: 387-390, 1972.

Bulos, B., Shukla, S., and Sacktor, B.: The rate of induction of the mitochondrial α-glycerolphosphate dehydrogenase by thyroid hormone in adult and senescent rats. Mech. Ageing Develop. 1: 227-231, 1972.

Carafoli, E., and Sacktor, B.: The effects of ruthenium red on reactions of blowfly flight muscle mitochondria with calcium. Biochem. Biophys. Res. Commun. 49: 1498-1503, 1972.

O'Brien, S. J.: On estimating functional gene number in eukaryotes. Nature New Biol. 242: 52-54, 1973.

Sacktor, B.: The bioenergetic properties and ultrastructure of mitochondria from flight muscle of aging blowflies. In Symposia Reports, 9th International Congress of Gerontology, Kiev, USSR. 1972, Vol. 2, pp. 84-86.

Sacktor, B.: Biological oxidations and energetics in insect mitochondria. In Physiology of Insecta. In press.

been unequivocally demonstrated by equilibrium dialysis experiments. At equilibrium, when ^{14}C-L-glucose, which is used as a marker for non-specific interactions as well as a measure of the included volume in the brush border preparations, is equally distributed in the inside and outside of the dialysis membrane, ^{3}H-D-glucose is concentrated many fold with the brush borders. The tubular cell luminal membrane has the capacity to bind D-glucose 100-times that obtained previously by short term Millipore filtration procedures, and values of 5 nmoles per mg membrane protein are obtained. Kinetic studies of the binding have characterized its time course, saturability (Km = 3-4 μM), reversibility, inhibition by phlorizin, and have determined the effects of Na^{+} as well as Ca^{2+} and Mg^{2+}.

Brush border membranes extracted with 1% Triton X-100 (approximately 16 mg per mg protein) for 10 min at 20° release about 90% of their protein, but the 105,000 x g pellet has the same affinity and capacity to bind glucose as has the intact membrane. Electron micrographs of the extracted membrane reveal complete disruption with no evidence of vesicular structure. This further distinguishes binding of glucose from uptake of the sugar into membrane vesicles.

Further investigations of the low affinity interactions of D-glucose with the brush border membranes indicate that the system is characterized best as being consistent with the hypothesis for a carrier mediated transport. Both the rates of influx and efflux are temperature dependent and the time courses at the different temperatures have been established. At 20°, the initial rate of D-glucose uptake increases essentially linearly with increasing concentrations of the sugar from 0.8 μM to the mM range, with progressive departure from linearity as the concentration exceeds 10 mM. However, the uptake as a function of concentration does not plateau at concentrations as high as 100 mM. Phlorizin significantly inhibits D-glucose efflux as well as influx, suggesting a mediated transport in and out of vesicles rather than simple binding where phlorizin would be expected to displace glucose and augment efflux. Prior loading of the brush border membranes with 50 mM D-glucose causes a 40% stimulation of the initial uptake of 25 mM D-glucose as compared to loading with 50 mM L-glucose or mannitol. Again, this effect, so-called countertransport or facilitated exchange diffusion, supports a transport phenomenon since in the case of binding the prior loading would be expected to inhibit subsequent uptake.

There is no significant difference between the uptake of 1 mM D-glucose by brush borders prepared and incubated in Na^{+} or choline media. However, using membranes prepared in 130 mM choline, the uptake is 50% greater when the incubation medium contains 65 mM choline + 65 mM Na^{+}, as compared to 130 mM choline or 65 mM choline + 65 mM Li^{+}. On the other hand, the uptake of D-glucose from a 65 mM choline + 65 mM Na^{+} medium is 50% greater if the preparation medium was 130 mM choline rather than 130 mM Na^{+}. These findings indicate that although Na^{+}, per se, is not required for transport of D-glucose by the brush border membranes, the rate of glucose transport by the membranes is greatly enhanced by a Na^{+} gradient from the outside to the inside of the membrane.

The bindings of D-galactose and several non-metabolizable sugars to isolated renal tubule luminal membranes have been estimated and the mutual interactions of these sugars and D-glucose have been examined. The order of binding of the various sugars is D-glucose$>\alpha$-methyl-D-glucose$>$D-galactose$>$2-deoxy-D-glucose$>>\alpha$-methyl-6-deoxy-D-galactose. The binding of 2-deoxy-D-glucose is Na^+-independent, and sensitive to phlorizin and sulfhydryl reagents. Binding of α-methyl-D-glucose has a Na^+-dependent component. The apparent Km for D-galactose binding is similar to that for D-glucose but the capacity for the binding of D-galactose is only 25% that of D-glucose. D-galactose and D-glucose mutually inhibit, competitively, the binding of the other. These results suggest that D-glucose and D-galactose have common or closely associated binding sites on the renal brush border membrane. The inhibitions of the high affinity binding of D-glucose by the non-metabolizable sugars are competitive and the apparent Ki values have been estimated. These findings on the bindings of various sugars to the isolated luminal membrane and their interactions with D-glucose at the membrane relate closely with other results on the uptakes of these sugars by renal cortical slices and by the intact kidney, in vivo. This strongly supports the view that studies of the interactions of sugars with isolated membranes are valid models for studying the mechanisms of active transport in the renal tubule.

Significance to Bio-medical Research and the Program of the Institute: This study has established a useful model to examine the molecular basis for the transport of substances across cell membranes. Physiological systems, such as renal, cardiac, neural activity and β-cell secretion, are intimately dependent on transport of ions and metabolites across plasma and intracellular membranes. These systems show marked functional changes with age. The paucity of information on the biochemical mechanisms for the transport of vital cell constituents frequently precludes a full understanding of normal physiological activities as well as those showing age-related changes. These investigations are designed to provide this needed information.

Proposed Course: Studies will continue to define the molecular basis of the transport of substances across cell membranes. The interrelationship and physiological roles of two processes, the binding of glucose to the isolated membranes and the carrier mediated transport of the sugar across the membrane remains to be clarified. The mechanisms of the transport of ions, especially Na^+, Ca^{2+} and phosphate, and of amino acids, as well as the interactions of the transport of these substances with that of sugars is of immediate concern. The isolated renal tubule luminal membrane is also used as a prototype for other membrane systems, e.g., the plasma membrane of the pancreatic islet cell. A study of the mechanisms whereby transport of sugars induces the secretion of insulin is planned. As fundamental information becomes available, this knowledge will be applied to the understanding of the functional changes that occur during the aging process.

Honors and Awards:

Dr. Sacktor was invited to present a lecture on "The Interaction of Sugars with the Renal Membrane" to the Philadelphia Physiological Society sponsored A. N. Richards Symposium on "Recent Advances in Renal Physiology and Pharmacology" at the Jefferson Medical College, Philadelphia, Pa.

Publications:

Sacktor, Bertram: The function of trehalase in the active transport of glucose in the mammalian kidney. In Piras, R., and Pontis, H. G. (Eds.): Biochemistry of the Glycosidic Linkage. PAABS Symposium. San Carlos de Bariloche, Argentina. 1972, Vol. 2, pp. 281-289.

Chesney, Russell, Sacktor, Bertram, and Rowen, Robert: The binding of D-glucose to the isolated luminal membrane of the renal proximal tubule. J. Biol. Chem. 248: 2182-2191, 1973.

Chesney, Russell, Sacktor, Bertram, and Kleinzeller, Arnost: The binding of phloridzin to the isolated luminal membrane of the renal proximal tubule and the interactions of phloridzin and D-glucose at the receptor sites on the brush border membrane. J. Biol. Chem., in press.

Chesney, Russell, and Sacktor, Bertram: Transport of sugar by isolated luminal membranes of the renal proximal tubule: binding of galactose and other sugars and their interactions with glucose. J. Biol Chem., in press.

Serial No. HD-GMA4(2)
1. Gerontology Research Center
2. Laboratory of Molelular Aging
3. Intermediary Metabolism Section
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Control of RNA Synthesis in Senescent, Developing, and Neoplastic Systems

Previous Serial No.: Same

Principal Investigators: David Gillespie

Project Description:

This project has been discontinued.

Publications:

Gillespie, D., Marshall, S., and Gallo., R.: RNA of RNA Tumour viruses contains Poly A. Nature New Biol. 236: 227-231, 1972.

Marshall, S., and Gillespie, D.: New rifampin-resistant mutant of E. coli. J. Bacteriology 110: 782-783, 1972.

Marshall, S., and Gillespie, D.: On the absence of poly U tracts from RNA isolated from RNA-containing viruses. Nature New Biol. 240: 43-45, 1972.

Patterson, D., and Gillespie, D.: A temperature-sensitive mutant of E. coli defective in the chain elongation step of protein synthesis. Biochem. Genetics 8: 205-230, 1973.

Patterson, D., and Gillespie, D.: The effect of elevated temperatures on protein synthesis in E. coli. J. Bacteriology 12: 1177-1183, 1972.

Patterson, D., Gillespie, S., and Gillespie, D.: Measurement of bacterial message RNA by RNA-DNA hybridization in formamide. Biochem. Genetics. In press.

Serial No. HD-GMA 8

1. Gerontology Research Center
2. Laboratory of Molecular Aging
3. Section on Intermediary Metabolism
4. Baltimore, Maryland

PHS-NIH
Individual Project Report
July 1, 1972 through June 30, 1973

Project Title: Investigations on the Biochemical Mechanisms of Hormonal Regulation of Metabolism

Previous Serial Number: None

Principal Investigators: Bertram Sacktor
Paul Insel

Man Years:

Total:	2.5
Professional:	1.2
Others:	1.3

Project Description:

Objectives: The broad objectives of this project are: (1) to understand the mechanisms by which metabolic processes are regulated by the action of hormones at the cellular and biochemical levels, and (2) to apply this fundamental knowledge to the understanding of the mechanisms whereby physiological control systems are altered during the aging process. Specifically, this new project has sought: (1) to localize in the renal cortex hormonally stimulated adenyl cyclase, (2) to study the binding of cyclic AMP to cortical membranes, (3) to identify the endogenous membrane-bound cyclic AMP stimulated protein kinase, (4) to identify and characterize the presumed phosphorylated enzyme, and (5) to determine the mechanisms by which the hormonally stimulated sequence of reactions regulate renal function.

Methods Employed: The primary preparation used in these studies is the plasma membrane of the renal cortical proximal tubule. As appropriate, modern biochemical procedures required to answer the questions posed are applied, modified, or developed.

Major Findings: An assay for adenyl cyclase, representing a modification of the Krishna technique, has been established for rabbit renal preparations. Kinetic parameters, estimations of recovery, identification of product, stability of enzyme, and appropriate concentrations of activators and inhibitors are now known for this preparation. Interestingly, renal adenyl cyclase differs from the hepatic enzyme in its insensitivity to GTP. Parathyroid hormone (synthetic 1-34), at 10 µg/ml, and catecholamines, most prominently isoproteranol, at 0.1 mM, effect as much as 400% and 140% stimulation of basic activity, respectively. The stimulations are not additive

with respect to F^- stimulation. Luminal membrane preparations from proximal tubules contain measurable activity, although the specific activity of the enzyme in this membrane relative to established enzyme markers is not enhanced.

Brush border membranes bind significant amounts of cyclic AMP, as determined by Millipore filtration and equilibrium dialysis procedures. The specific activity of the binding capacity is greatly enhanced in this isolated membrane preparation. Kinetic parameters for the binding have been partially established.

Significance to Bio-medical Research and the Program of the Institute: Knowledge of the biochemical mechanisms by which cellular processes are controlled by hormone action is of fundamental importance in understanding how homeostatic physiological systems operate. This information is necessary to understand the mechanisms by which metabolic control systems may be deleteriously changed during aging.

Proposed Course: It is planned to continue the elucidation of the mechanisms of hormonally-related processes in the renal tubule, as outlined in the Objective section.

Honors and Awards: None

Publications: None